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SECTION MEETINGS

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Electron Micrographs of the Agent of Feline Pneumonitis (Baker).

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The morphology of several viruses and rickettsiae has been studied with the aid of the electron microscope but no studies on any member of the psittacosis-lymphogranuloma-trachoma group have been published. Although the data presented here deal only with the agent of feline pneumonitis, similar micrographs have been obtained with agents of lymphogranuloma venereum, human pneumonitis* (S.F.) and psittacosis.*

The material for study was prepared from 10% or 20% suspensions of heavily infected yolk sacs which were shaken in Ringer's without beads for 20 minutes to liberate the agent from infected cells. From then on the procedure varied but the following gave the cleanest preparations. The supernate from shaking was twice centrifuged for 15 minutes at 2000 and at 2400 r.p.m. The agent was then sedimented twice at 15,000 r.p.m. for one hour and resuspended in Ringer's solution. After a third centrifugation at 15,000 r.p.m. for one hour the sediment was finally resuspended in the original volume of distilled water. This suspension was clarified finally at 2000 r.p.m. for 15 minutes and the supernate dried on collodion membranes in the usual manner.

As shown by the illustrations, the characteristic picture obtained is one in which a central more opaque mass is surrounded by a less opaque circular limiting membrane.

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* We wish to thank Dr. Francis Gordon for supplying suspensions of these two agents which had been inactivated by ultraviolet irradiation.

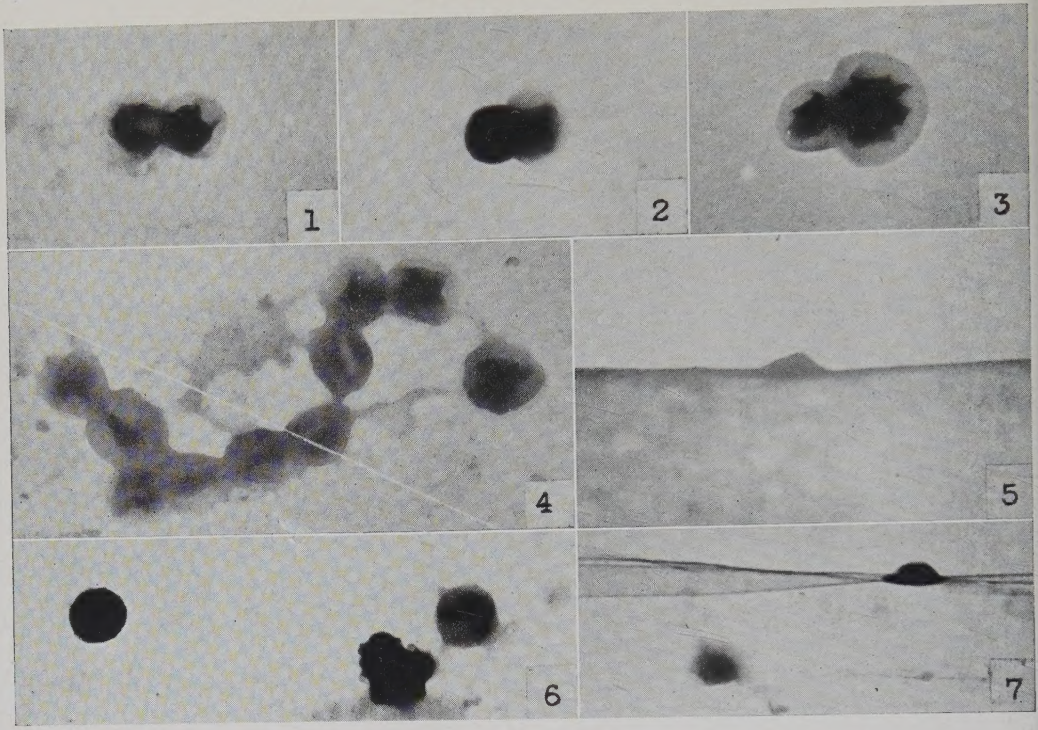

FIG. 1.
The elementary bodies of Feline Pneumonitis. Magnification 20,700 ×.

This appearance resembles closely that described for the rickettsiae[1-3] and certain bacteria,[4] less closely that shown by vaccinia[5,6] and not at all that of the smaller viruses. In the case of feline pneumonitis, the central substance is quite irregular internally as well as in outline (Fig. 1-4). The appearance suggests the irregularities of a wrinkled pea flattened on one side and in a few instances in which side views have been obtained due to rupturing of the collodion membranes, this opinion was confirmed (Fig. 5). In a very few instances dark bodies of uniform density are seen without any limiting membrane. It is not certain that these represent the bodies of the agent but since they are of the same general size and density as the central masses they may be undistorted bodies or bodies in which the substance of the limiting membrane has been lost (Fig. 2, 6). In some bodies showing the limiting membrane there was little distortion of the central mass. In side view such bodies appear like derby hats (Fig. 7).

The majority (51%) of the elementary bodies appear by direct measurement of electron micrographs to be between 440 and 490 mμ in diameter. The median diameter is 465 mμ, the average 455 mμ, and the range from 350 to 580 mμ. Such lack of uniformity is not seen with the smaller viruses. It seems probable that these diameters for the elementary bodies are larger than those existing in the viable forms. The flattened wrinkled pea form noted above suggests that the bodies resemble sacs filled with jelly which, settling down on the collodion membranes, lose their spherical shape and increase

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[1] Plotz, H., Smadel, J. E., Anderson, T. F., and Chambers, L. A., *J. Exp. Med.*, 1943, **77**, 355.

[2] Weiss, L. J., *J. Immunol.*, 1943, **47**, 353.

[3] Shepard, C. C., and Wyckoff, R. W. G., *Pub. Health Rep.*, 1946, **61**, 761.

[4] Mudd, S., and Anderson, T. F., *J. Am. Med. Assn.*, 1944, **126**, 561.

[5] Green, R. H., Anderson, T. F., and Smadel, J. E., *J. Exp. Med.*, 1942, **75**, 651.

[6] Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 259.

in diameter. Larger bodies, such as might correspond to initial bodies, are rare although at least one 770 mμ in diameter has been noted. There are two possible reasons for the paucity of initial bodies. They may be largely discarded with the low speed sediment or they may fragment, at least to the extent of losing their capsular material, in the process of repeated differential centrifugation. There is evidence that both occur. Thus examination with the light microscope of low speed supernates of preparations, originally rich in initial bodies, show complete disappearance or marked decrease in numbers—to between 1% and 2%. Such supernates are then further treated, by repeated high speed sedimentation and resuspension, and those showing any initial bodies suffer a further 90% decrease.

Pairs, chains and groups of elementary bodies are to be found (Figs. 1-4). Fairly dense intercellular bridges stretching between bodies are not infrequent and may be enclosed by limiting membranes (Fig. 4). The picture is reminiscent of that shown by certain bacteria.[4]

Summary. Electron microscope studies of the agent of feline pneumonitis suggest that the elementary bodies in nature have properties similar to those of jelly-filled sacs which in course of preparation for examination in the microscope settle to a form similar to a wrinkled pea with one flattened side. Such distorted bodies are approximately 465 mμ in diameter. They have several characteristics which resemble rickettsiae and bacteria.

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Pathogenizing Effect of Different Carbohydrates on *Eberthella typhosa*.

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It has long been known that the pathogenicity of bacteria for laboratory animals can be enhanced by addition of certain substances to the injected suspensions (cf. references in papers of McLeod,[1] Ercoli *et al.*[2]). Several investigators have used carbohydrates for this purpose. No systematic study of the relation between carbohydrate structure and pathogenizing potency has, however, been reported. The present communication reports experiments which form part of an investigation designed to clarify the chemical basis of pathogenizing activity in the carbohydrate group.

The experiments define the activity of selected sugars and polysaccharides as pathogenizing agents for *E. typhosa* in the mouse. Special attention is directed to the pathogenizing activity of several levans, including 2 natural sources and a preparation synthesized from sucrose by a cell-free system. The information obtained is inadequate to define finally in a positive sense the features of molecular pattern which determine pathogenizing activity in the carbohydrate group. It suffices, however, to show that several features of the chemical structure and physical character of a carbohydrate are unimportant to its rôle as a pathogenizer.

Test organism. E. typhosa (0901) served as the test microorganism. The bacteria were grown in plain broth at 37°C, the incubation lasting 24 hours.

Test of pathogenizing activity. The experiments were designed to show whether intraperitoneal injection of carbohydrate into a mouse has a pathogenizing effect. White mice of laboratory stock served as the experimental animal. The mice were at the time of the tests 4-5 weeks old and averaged 18 g in weight. The animals were injected intraabdominally with 0.5 cc of a suspension which consisted of a mixture of 0.05 cc of a selected dilution in broth of a 24-hour broth culture and 0.45 cc of a sterile-aqueous solution of the tested pathogenizing substance. Where no pathogenizing effect was observed, the carbohydrate concentration was increased in successive tests up to a concentration of 10% or until a concentration which gave a solution too viscous to be injected was reached. Where a pathogenizing effect was obtained the number of injected bacteria was decreased by power of 10 in successive tests until a nonlethal dose was reached. Groups of 5 mice were used at each assay level. The findings are recorded in terms of the quotient—number of animals which died within 48 hours after injection/5 (total number of mice in group). The pathogenizing activity is expressed in terms of the quotient, MLD of broth culture without added pathogenizer/MLD of broth culture in presence of added pathogenizer. In order to exclude error due to accidental death, the MLD is defined as that dose which produces a mortality rate of at least 2/5. The MLD of the broth cluture without added pathogenizer was found to be 0.4 cc. In repeats of assays of the pathogenizing potency of different substances, the maximum variation in mortality between replica tests was ± 1/5. Values of pathogenizing potency were reproducible within the limits of one logarithm integer of the broth dilution.

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[1] McLeod, Ch., *Am. J. Hyg.*, Sec. B, 1941, **34**, 41 and 51.

[2] Ercoli, N., Lewis, M. N., and Harker, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 273.

Materials. Sugars: pure crystalline preparations.* Galactomannan:† a preparation from carob seed. Mannan: prepared from carob galactomannan by selective hydrolysis according to the method of Wailew and Gortner.[3] Dextran: prepared in powder form from sucrose broth culture of *Leuconostoc mesenteroides* by repeated precipitation in alcohol with stirring in a Waring blendor. Levans: prepared in powder form from sucrose cultures of *Aerobacter levanicum* and *Bacillus subtilis* as described by Hestrin *et al.*[4] Cellulose I: fibril suspension prepared from cultures of *Acetobacter xylinum* as described by Aschner and Hestrin.[5] Cellulose II: acid-degraded cotton cellulose prepared by exposure of one part ether-extracted cotton wool to the action of 9 parts 60% sulfuric acid for 10 minutes at room temperature. The suspension after dilution and neutralization was dialyzed and obtained in the form of a powder by evaporation over a boiling water bath. Other materials employed: glycogen,‡ inulin,§ gum acacia,‖ agar powder,¶ mucin,** kaolin,†† rice starch, soluble starch, and pectin.‡‡

Survey of pathogenizing activity of different carbohydrates. The following carbohydrates were found to be entirely lacking in pathogenizing activity in a concentration range of 0.2-10%: pentoses—arabinose and xylose; hexoses—glucose, fructose and galactose; oligosaccharides—maltose, trehalose, lactose, cellobiose, sucrose, and raffinose; polysaccharides — galactomannan (0.2%), soluble starch (5%), rice starch (5%) and pectin (5%). The MLD in the presence of these carbohydrates except in the case of pectin was equal to that found in absence of carbohydrate. Pectin was found to be bactericidal at a concentration of 5%.

Polysaccharides which showed measurable or marked pathogenizing activity under the conditions of the test are listed in order of decreasing activity in Table I. On the basis of the maximum pathogenizing effect (pathogenizing potency) obtained 3 classes of carbohydrates can be distinguished: (a) pow-

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* British Drug Houses, London.

† Kindly supplied by Dr. E. Simon, Daniel Sief Research Institute.

[3] Wailew, B., and Gortner, R., *Arch. Biochem.*, 1943, 1, 325.

[4] Hestrin, S., Avineri-Shapiro, S., and Aschner, M., *Biochem. J.*, 1943, **37**, 450.

[5] Aschner, M., and Hestrin, S., *Nature*, 1946, **157**, 659.

‡ Rhône-Poulenc, Paris.

§ British Drug Houses, London.

‖ Pharm. central de Belgique.

¶ May and Baker, London.

** Burroughs Wellcome, London.

†† Merck, Darmstadt.

‡‡ Commercial source unknown.

TABLE I.
Pathogenizing Activity of Polysaccharides in Presence and Absence of Kaolin.

	Pathogenizing activity expressed as ratio: MLD without pathogenizer/MLD with pathogenizer						
	Concentration of carbohydrate						
Substances tested	0.2%	0.3%	0.5%	1.0%	2.0%	5.0%	10.0%
Agar	—	$8\text{x}10^{3}$	—	—	—	—	—
” with kaolin 1%	—	$4\text{x}10^{3}$	—	—	—	—	—
Levan *(A. levanicum)*	—	$8\text{x}10^{2}$	$8\text{x}10^{3}$	$8\text{x}10^{4}$	$8\text{x}10^{5}$	—	—
” with kaolin 1%	—	—	—	T	T	T	T
Levan *(B. subtilis)*	—	—	—	$8\text{x}10^{3}$	$8\text{x}10^{4}$	—	—
” with kaolin 1%	—	—	—	$4\text{x}10^{3}$	$4\text{x}10^{4}$	—	—
Mucin	—	—	—	—	—	$8\text{x}10^{4}$	$8\text{x}10^{5}$
” with kaolin 1%	—	—	—	—	—	T	T
Dextran *(L. mesenteroides)*	—	—	—	$8\text{x}10^{2}$	$8\text{x}10^{3}$	—	—
” with kaolin 1%	—	—	—	—	$4\text{x}10^{3}$	—	—
Cellulose I *(A. xylinum)*	—	—	—	—	8	—	—
” with kaolin 1%	—	—	—	—	$4\text{x}10^{2}$	—	—
Cellulose II (Cotton)	—	—	—	—	—	80	—
” with kaolin 1%	—	—	—	—	—	$4\text{x}10^{2}$	—
Gum acacia	—	—	—	—	—	1	80
” with kaolin 1%	—	—	—	—	1	4	$4\text{x}10^{3}$
Mannan (carob)	—	—	—	—	—	8	—
” with kaolin 1%	—	—	—	—	—	4	—
Glycogen	—	—	—	—	1	1	8
” with kaolin 1%	—	—	—	—	40	40	$4\text{x}10^{3}$
Inulin	—	—	—	—	1	1	4
” with kaolin 1%	—	—	—	—	1	40	$4\text{x}10^{2}$
Dextrin	—	—	—	—	—	—	1
” with kaolin 1%	—	—	—	—	—	—	40

— = No test.
1 = MLD not lowered; 8 = MLD 8-fold lowered, etc.
T = Toxic action in absence of bacteria.

erful pathogenizers; (b) moderate pathogenizers and (c) nonpathogenizing carbohydrates. Class (a) includes 2 extracellular bacterial polysaccharides (levan and dextran), mucin and agar-agar. Members of this class showed high activity as pathogenizers also at fairly low concentration. Class (b) comprises the 2 cellulose preparations, gum acacia, glycogen, mannan and possibly should also include inulin. Class (c) comprises all the tested crystalline sugars and several of the polysaccharides (starch, galactomannan). It may be noted that the above classification of carbohydrates on the basis of pathogenizing potency is consistent with subdivision of the carbohydrates on the basis of other criteria of their pathogenizing action as described in following sections.

Copathogenizing effect of kaolin. Kaolin alone at a concentration of 1% produced no significant pathogenizing effect, the MLD being in absence of kaolin 0.4 cc and in its presence 0.2 cc. Kaolin is, however, in certain cases a powerful copathogenizer, *i.e.* it enhances the pathogenizing potency of carbohydrates which also show pathogenizing activity in its absence. The influence of kaolin on the pathogenizing potency varies consistently with the classification of carbohydrates already described. Kaolin exerts a marked effect on the pathogenizing activity of carbohydrates of class (b), but has little or no effect in class (c) and (a).

Effect of pathogenizer concentration. The effect of pathogenizer concentration has been investigated in the following: levan, mucin, gum acacia, inulin and glycogen. It is evident from the values shown in Table I that the pathogenizing activity increases at an increasing rate as the concentration of pathogenizer is increased. There is in every case a threshold level below which no activity can be found. The threshold varies markedly in the different carbohydrate classes. It is relatively low ($<2\%$) in (a), and fairly high (5-10%) in (b). The existence of the threshold places doubt on the general significance of negative results which may be obtained when possible pathogenizers are tested at low concentrations.

Pathogenizing activity of synthetic levan preparation. The observations on the pathogenizing activity of levan from 2 different bacterial sources suggest that levan from *A. levanicum* has significantly greater pathogenizing potency than levan from *B. subtilis.* This observation is of particular interest in view of the fact that no chemical or serological difference between different bacterial levans has hitherto been detected.[6,7] The pathogenizing action of levan prepared by the action on sucrose of a cell-free enzyme solution was therefore undertaken.

In preliminary experiments it was found that levan-free levan-sucrase solution prepared from *A. levanicum* by autolysis as described by Hestrin *et al.*[4] is toxic to mice in a dosage of 0.1 cc. Since the toxin is heat-stable and derives from a Gram-negative organism, it is probably to be regarded as endotoxin. The toxin of the enzyme preparation is precipitated by alcohol. It accompanied levan in the alcohol precipitate when levan was formed from sucrose added to the enzyme solution. Methods accordingly were sought which would permit the preparation of a nontoxic synthetic levan preparation. Two suitable methods are described below.

In one case, levan was synthesized from sucrose by incubation with levan-free levan-sucrase at 37°C for 2 days in a mixture of the following composition: 100 cc enzyme solution, 100 cc acetate buffer pH 5.0, 100 cc 15% sucrose. Toluol was added to maintain sterility. The material was precipitated with 2 parts alcohol, purified by repeated precipitation with alcohol, and dried in a vacuum desiccator. About 1.2 g of dry material was thus obtained, whereas a control mixture of enzyme and buffer without sucrose afforded 0.3 g by the same process. The yield of levan was therefore 0.9 g. To remove possibly present endotoxins the 2 products were treated according to Furth and Landsteiner[8] with hot 0.5 N NaOH for 30 minutes. The solutions were neutralized and dialyzed against tap water in cellophane bags for one day. The dialyzed solutions were precipitated with 2 volumes of alcohol. The precipitates were dried and aqueous solutions of 1 and 2% concentration were set up. Solutions of the enzyme-buffer mixture product proved to be nontoxic and nonpathogenizing, whereas the solutions of the enzyme-buffer-sucrose product, *i.e.* the synthetic levan preparation, showed no toxicity but high pathogenizing activity. The pathogenizing activity rated 8×10^2 and 8×10^3 at the concentration levels 1% and 2% respectively.

In a second case, advantage was taken of the fact that dilution improves the levan yield per unit of enzyme employed.[9] The reaction mixture was composed as already described except that the enzyme solution was diluted 10-fold. The yield of levan was decreased by only one-half by this 10-fold decrease of the enzyme. A levan preparation was obtained which showed no toxic effect in 2% solution even in absence of alkali pretreatment.[8] The pathogenizing activity was found to rate 8×10^2, 8×10^2 and 8×10^3 at the concentration level 0.5, 1.0 and 2.0% respectively.

It may be concluded therefore that levan synthesized *in vitro* by the action of cell-free *Aerobacter* levan-sucrase on sucrose exhibits a marked pathogenizing activity independent of the presence of any alkali-labile toxic component in this preparation. The "synthetic" levan shows slightly less activity than levan prepared from a living *Aerobacter* culture.

Discussion. The experiments presented show that pathogenizing activity towards *E. typhosa* is not uncommon in polysaccharides. Of a total of 15 polysaccharides investigated, high pathogenizing potency was found in 5, moderate activity in 6, and little or no activity in the remainder. On the other hand,

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[6] Genghof, D. S., Hehre, E. J., and Neill, J. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 339.

[7] Hehre, E. J., Genghof, S. D., and Neill, J. M., *J. Immunol.*, 1945, **51**, 5.

[8] Furth, J., and Landsteiner, K., *J. Exp. Med.*, 1928, **47**, 171.

[9] Avineri-Shapiro, S., and Hestrin, S., *Biochem. J.*, 1945, **39**, 167.

not one member of a group of 10 oligosaccharides and simpler sugars examined showed pathogenizing activity. It is appropriate in the light of these findings to inquire what physical and chemical properties in a carbohydrate determine its activity.

The hypothesis has been presented that viscosity is an overriding determinant of the pathogenizing activity of an injected substance. Experiments by several investigators have tended, however, to throw doubt on this conclusion. The experiments here reported confirm this criticism. Among the carbohydrates examined by us, no consistent relationship between viscosity and pathogenizing activity can be observed. In the polysaccharide group with marked pathogenizing activity, dextran with a viscosity at a concentration of 2% about 10 times that of levan shows far less pathogenizing activity than does levan at the same concentration. Again, in the group of polysaccharides with low pathogenizing activity, a pathogenizing potency which is roughly constant is associated with widely fluctuating viscosities. Finally, it may be noted that the inactive polysaccharide group includes several substances of very high viscosity (galactomannan, mannan, starch). It is clear, therefore, that viscosity is not an overriding determinant of pathogenizing activity and that it does not *per se* confer pathogenizing activity on an injected substance.

Since a pathogenizing activity which is absent in simple sugars and oligosaccharides can be present in their polymers, it is suggested that pathogenizing activity is a function of patterns which are specific to the colloidal state. It is significant in this respect that different polysaccharides with a common hexose unit and similar features of chemical structure can present widely different pathogenizing activity (levan from *A. levanicum* vs. levan from *B. subtilis* vs. inulin; starch vs. dextran, etc.)

Pathogenizing activity seems to be dependent only in a minor degree on the structure, configuration and manner of linkage of the individual repeating unit or on the presence of polar groups in a carbohydrate polymer. Thus it has been found that either high or moderate pathogenizing activity may be manifested by simple polysaccharides of such groups as glucan (dextran, cellulose preparations, glycogen), fructan (levans, inulin), and mannan (carob preparation) and by complex polysaccharides with polar groupings (agar, gum acacia, mucin). Our pathogenizing carbohydrates include both α and β linkage types, furanosidic as well as pyranosidic units, and intersaccharidic C-C linkages of the 1-4 (cellulose, glycogen), 1-6 (dextran), 2-1 (inulin) as well as 2-6 (levan) types.

In an investigation of the pathogenizing potency of different fractions of mucin, Anderson and Oag[10] found that whereas marked activity is shown by protein fractions no pathogenizing activity is presented by the purified carbohydrate fraction of mucin. This result suggests that the pathogenizing activity of certain carbohydrates may be due to the presence of foreign substances in these preparations. In view of the fact that pathogenizing activity is manifested by polysaccharides from widely different sources, this possibility must be considered as most unlikely. In the case of levan it has been possible to exclude it. The demonstration of pathogenizing activity in a levan obtained in defined reaction conditions by the action of cell-free enzyme on pure sucrose affords proof that pathogenizing activity is an intrinsic property of levan.

Summary. The pathogenizing action of 15 polysaccharides and of 11 simple sugars and oligosaccharides on *Eberthella typhosa* in the mouse has been described. High pathogenizing activity was shown by the following carbohydrates: levan, dextran, mucin, and agar-agar. Moderate activity was shown by the following: cellulose preparations, gum acacia, glycogen and mannan. Inclusion of kaolin in the injected suspension enhanced the pathogenizing action of this group. Levans from 2 bacterial sources *Aerobacter levanicum* and *Bacillus subtilis* showed different degrees of pathogenizing activity. Levan synthesized *in vitro* by the

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[10] Anderson, C. G., and Oag, R. K., *Brit. J. Exp. Path.*, 1939, **20**, 25.

action of cell-free *Aerobacter* enzyme showed marked pathogenizing activity. The pathogenizing activity must be, therefore, an intrinsic property of this levan.

It is suggested that the pathogenizing activity of a polysaccharide is a function of patterns which are specific to the colloidal state. The activity depends only in minor degree on the structure, configuration and manner of linkage of the individual repeating unit or on the presence of polar groupings in the carbohydrate polymer. There is no consistent relationship between the viscosity of the injected carbohydrate solution and the pathogenizing activity which it manifests.

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Electroshock Therapy by Stimulation of Discrete Cortical Sites with Small Electrodes.*

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Among the many phenomena observed during and following electroshock therapy are convulsion, loss of memory and profound stimulation of the autonomic nervous system. By using small electrodes (8 mm in diameter as contrasted to conventional electrodes of 5 cm^2) it was found that no convulsion resulted when the stimulus was applied through regions some distance from the motor strip, although the current used was sufficient to produce convulsions when applied to the motor strip itself. The conventional shock machine was used in the experiment. Fairly accurate control of current was possible by measuring the resistance of the subject.

Some degree of localization of the stimulation was possible. Observations made following stimulation of several chosen sites indicated that stimuli over the various regions produced different results. Correlating these observations with clinical results and information concerning other organic treatments used in mental disorders, has made possible some speculation concerning the factors which make electroshock treatment effective. It has also offered directions for further investigations into the mechanism of the psychoses.

Sites for stimulation through the frontal area were chosen in accordance to external skull landmarks. Site A was high on Area 4 (Brodmann) just posterior to the interauricular line. Site B was roughly through Brodmann's Area 11. Site C was roughly through Brodmann's Areas 9 and 10. Site D was a point midway between the orbit and auditory canal and 1 cm above the upper border of the zygomatic process. (Brodmann Area 38).

Observations of results following stimulation of the selected sites will be presented according to:

(A) Motor phenomena
(B) Autonomic nervous system phenomena
(C) Disturbance of intellectual function
(D) Clinical results

(A) Motor phenomena. Stimulation of Site A or Area 4 of Brodmann resulted in a typical tonic and clonic convulsion lasting 40 seconds to one minute. Stimulation of the other sites produced with considerable regularity a single extension "jerk" of all extremities.

(B) Autonomic nervous system phenom-

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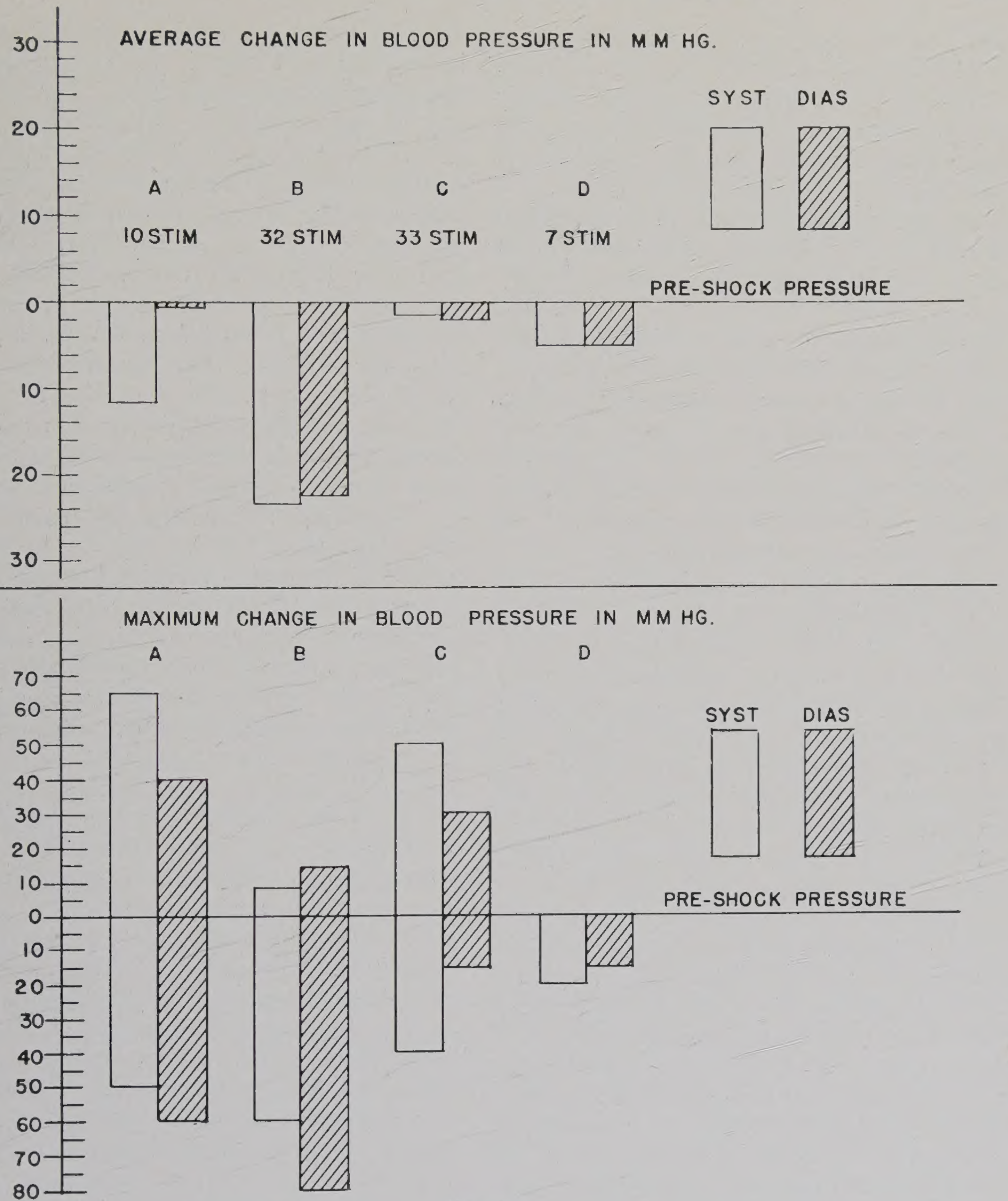

FIG. 1.
A. Average change in blood pressure. B. Maximum change in blood pressure.

ena. 1. Blood pressure (Fig. 1A and 1B).

Site A—Ten stimulations with convulsion. There was a rise of systolic and diastolic pressure in 2 cases and a fall in 8. Average systolic change was —11.5 mm; extremes +65 mm and —50 mm. Average diastolic change —0.5 mm; extremes +40 mm and —60 mm.

The time at which blood pressure was recorded in relation to the first deep respiration probably accounts for the inconsistent finding of 2 rises and 8 falls in pressure. With the cessation of respiration accompanying the tonic and clonic movements, there is a rise of pressure. With the first deep breath following the convulsion there is a rapid fall.

Site B—32 stimulations; blood pressure increased in 2, dropped in 30; average systolic change —23.6 mm; extremes —60 mm and +8 mm. Average diastolic change —22.3 mm; extremes —80 mm and +14 mm.

Site C—33 stimulations; average systolic change —1.4 mm; extremes +50 mm and —40 mm. Average diastolic change —2.0 mm; extremes +30 mm and —15 mm.

Six patients were stimulated in both Site B and Site C. In 3 of these cases Site B stimulation produced a fall, whereas C caused a rise. In the other 3 patients a drop was recorded after stimulation over both Site B and Site C. The fall after stimulation of Site B was, however, considerably greater.

Site D—7 stimulations. On 5 occasions there was no change. Twice the pressure dropped. Average change —5 mm in both systolic and diastolic pressure; extremes —20 mm systolic and —15 mm diastolic.

Summary. Stimulation at Site B produced a marked depressor effect after 30 of 32 stimuli. The effect of stimulation at Site C was not so consistent, the pressor response being noted with about the same frequency as the depressor. Over Site D there was no change in pressure following 70% of the stimulations. The effect of motor response on blood pressure changes is not important here as it was the same with excitation of each of the 3 sites.

2. Pulse rate. Recordings of pulse following stimulation of Site A are of no value because of the accompanying convulsion. After stimulation of Site B in 10 patients the rate was slowed in 7 and increased in 3. In contrast, after stimulation at Site C the rate speeded in 7 and slowed in 3. The rate was consistently slowed in 5 cases stimulated over Site D.

3. Pupillary response. Observations were made on 5 patients after stimulation at Site A and, on every occasion, the pupils were observed to dilate. Following stimulation at Site B, contraction was noted in 26 cases and dilation in but 3. Stimulation at Site C produced contraction in 22 cases and dilation in 23. Of 20 cases stimulated over Site D, 15 showed contraction and 5 dilation.

4. Peripheral vasomotor reaction. During the convulsion following Site A stimulation, the vasomotor reactions were not consistent. Usually, however, there was pallor immediately after stimulation, followed in a few seconds by a generalized flush. Toward the end of the convulsion cyanosis set in; but was followed again by flush with the initial deep respiration. One hundred ten stimulations over Site B were followed in every case by marked flushing of the skin. Almost invariably lacrimation was also noted. In 50 observations after stimulation at Site C, the flush was noted in two-thirds, but in the other one-third (15 cases) there was pallor of the skin. In 5 of the 15 cases which showed pallor, there was also profuse sweating. Site D stimulation produced very little change in peripheral vasomotor status.

5. Respiration. Slowing of respiratory rate, and often a period of prolonged apnea with mild cyanosis, was noted consistently following Site D stimulation. As Area 6B (Brodmann) was approached, this became more pronounced. Bucy[1] stimulated this area during a craniotomy on a patient and found that respiration temporarily ceased. His paper also refers to other work on cortical respiratory centers. Our findings support his conclusion that this area is a respiratory inhibitory center. It must, however, be considered that the apnea may have resulted from spread to medullary centers.

The only conclusion that can be drawn from these observations of autonomic phenomena occurring with focal stimulation of the brain through the skull is that there is a cortical autonomic level which when stimulated does exert a strong effect. The finding of different responses to stimulation of the various sites suggest localized areas of autonomic representation. However, by such diffuse stimulation one cannot hope to accurately localize autonomic function.

(C) Disturbance of intellectual function. Immediate effects: Following conventional electroshock treatment the patient remains dazed for 15 to 20 minutes. He has a blank, far away stare in his eyes and does not respond to the usual stimuli. He is often restless, and thrashes about aimlessly in bed. At times there is rather violent motor activity. This marked confusion usually clears in 30 minutes to a few hours.

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[1] Bucy, P. C., and Chase, T. J., *J. Nervous and Mental Diseases*, 1936, **84**, 156.

A review of the literature indicates that about 80% of cases develop a more lasting memory impairment which remains a few weeks to several months. Several authors[2,3] have suggested that some intellectual impairment may be irreversible. A few cases[3] are cited in which disturbances of memory remain 2 to 3 years following treatment.

Following stimulation over Site A (motor strip with convulsion) the recovery was more rapid. Patients could answer simple questions in less than 5 minutes. They did not develop the same dazed, far away look nor the marked hyperkinesia.

After stimulation of Sites B and C patients complained of anxiety and discomfort and were reluctant to continue treatment. There was no evident intellectual impairment.

Stimulation at Site D produced amnesia for several minutes. The patients appeared dazed and confused like those who had received conventional shock. The effect was, however, of shorter duration.

(D) Clinical results. Site A—(motor strip)—Results from stimulation of this area were essentially the same as with conventional therapy (*i.e.,* it was very effective in the treatment of affective psychoses—involutionals and manic depressive - depressed); with equivocal results in manic depressive—manic and schizophrenia. This method of administering convulsion did, we believe, possess an added advantage. Inasmuch as there was little memory loss and confusion, it was possible to administer psychotherapy in conjunction with electroshock therapy.

A case history will be briefly outlined to illustrate this: A 27-year-old, white female with a history of a mild depression 2 years previously, from which she spontaneously recovered in the course of 6 months, witnessed the bombing of Pearl Harbor during which her fiance was killed. After repatriation to the U. S. she spent 2 months nursing her fiance's mother through a nervous breakdown. She then took a job as stenographer at the San Diego Naval Base so as to be close to his friends. In an effort to forget she began to drink heavily and her morals became lax. (She had been raised strictly in the Catholic faith and attended Catholic boarding schools). Eventually pregnancy resulted and, taking the advice of a friend, she had an abortion performed. It was while convalescing from this that her psychosis developed, taking the form of schizo-depression with auditory hallucinations, delusions of persecution, self-abasement and attempted suicide. She entered the Pennsylvania Hospital a few days later and after the preliminary workup, electroshock treatments were begun. After 3 or 4 treatments she was so confused as to be unable to give her own name. Although all symptoms of psychosis disappeared several additional treatments were given. Approximately 7 to 10 days after the last treatment the confusion began to subside but, as her memory returned and before it was possible to begin psychotherapy, the symptoms of the psychosis returned. This procedure was repeated twice more, *i.e.,* treatment with improvement while confused, followed by relapse as memory returned. After the third relapse, shock treatment was instituted with small electrodes over the motor strip. No confusion or memory loss resulted and it was possible to administer psychotherapy shortly after the conclusion of the convulsion. The patient talked out her problems and remained well.

Site B stimulation was considerably less effective than was stimulation which produced a generalized convulsion as with Site A. Some patients, however, were benefited clinically and one made a complete recovery, but not until 20 to 25 treatments or many more than the customary number were given.

Site C—Results of stimulation of this area were essentially the same as those obtained from stimulation of Site B.

Site D—Stimulation of this area alone produced no clinical improvement. Analysis of data thus far accumulated suggests that:

1. Amnesia is not necessary to produce clinical improvement.

2. Respiratory complications can be les-

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[2] Levy, N. A., Serota, H. M., and Grinker, R. R., *Arch. Neurol. and Psych.,* 1942, **47**, 1009.

[3] Brody, M. B., *J. Men. Sci.,* 1944, **90**, 777.

sened by avoiding Area 6B (Brodmann), the cortical respiratory inhibitory center.

3. Some benefits are derived from stimulation through the frontal lobes without convulsion, although results are considerably below those achieved with convulsion.

4. Stimulation through Site D (temporal cortex, basal ganglia and hypothalamus) which produced memory loss but little effect on the autonomic nervous system (except pupillary responses) resulted in no clinical improvement.

Frontal lobe stimulation (at Sites B and C) produced some clinical improvement. It was, however, considerably less effective than motor strip (Site A) stimulation. The autonomic nervous system was affected in stimulation of all these sites. The autonomic cortex is reported to extend from the central sulcus anteriorly to the frontal pole.[4,5] There is a marked difference in the duration of the effects of the stimulus in these 2 regions. When Sites B and C were stimulated the effect was for the duration of the stimulus only (0.3 to 0.6 second). When Site A was stimulated producing a convulsion there was a spread of the excitation over the entire motor strip and it continued throughout the convulsion (45 sec. to 1 min.)

It seemed possible, therefore, that profound excitation of the autonomic nervous system might be the reason for clinical improvement. If this were the case, it might be possible to obtain autonomic stimulation and, therefore, clinical improvement without convulsion by repeated stimulation through the frontal lobe anterior to the motor strip.

In an effort to further investigate this assumption repeated stimuli were given through the frontal lobes anterior to the motor strip. In this way more prolonged autonomic stimulation could be obtained, still without convulsion, and if the forementioned speculations were correct, that clinical results were obtained from excitation of the autonomic nervous system, our percentage of cures should have increased.

Six stimuli about 10 seconds apart were administered throughout the frontal area. The first patient resented this treatment and refused to permit it because of the intense anxiety produced by each shock (as mentioned above in discussion of results of Site B and C stimulations). To overcome this complication we first stimulated Site D. Amnesia resulted and, thereafter, the patient had no recollection of the treatment and submitted uncomplainingly (much as in conventional therapy) to further stimulation at other sites.

Before results are discussed it should be pointed out that even with 6 shocks of 0.5 second each, the duration of autonomic excitation is considerably less than that seen in the generalized convulsion (45 sec. to 1 min.)

Table I gives a comparison of the clinical results obtained with stimulation of the various sites. The one manic depressive-depressed case treated unsuccessfully with multiple stimuli was subsequently given a full course of conventional shock treatment with no improvement. He was transferred to a state institution.

Case history to illustrate this procedure. A 69-year-old, white female. Prepsychotic personality; meticulous, rigid. Married but not happy; 2 children. Because of financial difficulties after husband's death, lived with sister for 5 years before onset of illness. Did not get along with sister.

Present illness. Ostensibly precipitated by a flood which damaged her home in January 1943; became extremely agitated and depressed; threatened suicide; self-accusatory; believed herself destitute and penniless; entered Pennsylvania Hospital March 1943.

Physical examination. 1. Old fracture of right hip with shortening of right leg.

2. Hypertrophic arthritis.

X-ray of spine: Degenerative changes and narrowing of discs.

X-ray of hip: Old osteochondritis with obliteration of the joint space.

Orthodox shock therapy was advised against by the roentgenologist. The family could ill afford prolonged hospitalization in

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[4] Kennard, M. A., *Illinois Monographs in the Medical Sciences*, 1944, 4, 295.

[5] Fulton, J. F., *Physiology of the Nervous System*, second edition, New York, Oxford University Press.

TABLE I.
Summary of Clinical Results in Electro-shock Treatment with Small Electrodes.

	Manic Depressive-Depressed					Involutional Melancholia				
Site	A	B	C	D	Multiple stimuli	A	B	C	D	Multiple stimuli
Well	8 63%	1 12.5%	0	0	0	5 83%	1 16%	1 16%	0	2 50%
Improved	2 27%	4 50 %	3 50%	0	0	1 17%	2 34%	2 34%	0	1 25%
No change	1 10%	3 37.5%	3 50%	2 100%	1 100%	0	3 50%	3 50%	2 100%	1 25%
Total No. of cases treated	11	8	6	2	1	6	6	6	2	4

a private institution and felt any therapy was preferable to state hospitalization.

Treatment was begun in May, 1943. She received 7 stimuli on each of 34 consecutive days, exclusive of Sunday, the first being directed through Site D so as to produce amnesia for the treatment. At the end of these periods all symptoms of her illness subsided and shortly she was discharged to her daughter. Regular follow-up letters indicate she continues to remain well.

Analysis of results of multiple stimuli. Although our cases are too few in number to permit definite conclusions the evidence is sufficient to make the following statements:

1. With multiple stimuli to that part of the frontal cortex that has autonomic representation the results appear to be better than with single stimulation.

2. The increased percentage of cures tends to support our hypothesis that stimulation of the autonomic nervous system may play a large role in the clinical results in electroshock therapy.

Other evidence to indicate the role of the autonomic nervous system in mental disorders.

1. The suggestion emphasized by these procedures, that the autonomic nervous system plays a major role in the affective mental disorders, is supported by frequent reports of findings of altered autonomic function in these psychoses. For example, in the involutional case there is insomnia, agitation, often elevated blood pressure, inability to eat, etc. Altered autonomic functions in depressed cases are also well known.

2. Clinical results reported with electronarcosis are at least as favorable as those reported with electroshock in the treatment of the affective disorders and are considerably better in the treatment of schizophrenia. In electronarcosis a smaller current than that used in electroshock is administered over a prolonged period. (60-cycle a.c. machine—initial current of 150 to 250 milliamperes for 30 seconds then decrease to level which permits respiration *i.e.*, 45 to 90 m.a. for up to 20 minutes). Frostig *et al.*[6] describe autonomic effects, motor phenomena and loss of consciousness with this procedure. The motor effects are perhaps less pronounced than with electroshock. There is an initial extensor spasm, then a few tonic and clonic movements, after which all variation between narcosis and marked hyperkinesia are seen for the duration of the treatment. The autonomic effects are quite variable. They are much more prolonged than with electroshock (dependent upon duration of treatment). The electrodes used in this treatment are 5 cm x 5 cm and the placement is over the temporal region. An extensive area of the brain is, therefore, stimulated much as in conventional electroshock.

3. Lobotomy, another effective form of treatment of the psychoses is directed toward affecting the autonomic nervous system. By this procedure pathways are cut which link the prefrontal cortex with the thalamus and lower centers, thereby isolating the autonomic cortex. Watts and Freeman[7] in describing

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[6] Frostig, J. P., Van Harreveld, A., Reznick, S., Tyler, D. B., and Wiersma, C. A. G., *Arch. Neurol. and Psych.*, 1944, **51**, 232.

[7] Freeman, W., and Watts, J. W., *Tr. N. Y. Acad. Sci.*, 1944, **6**, 284.

the operation state that immediately there is a disappearance of nervous tension. Although memory is intact and conflicts still exist, they cause little concern. They report their patients are able to formulate ideas but lack the accompanying emotional drive that is necessary to put them into effect. As a result their productivity is usually markedly impaired.

Summary. By using small electrodes in electroshock treatment it was possible to localize the stimulation sufficiently to separate some of the many phenomena that occur with conventional electroshock therapy. The small amount of data thus far accumulated seems to indicate: (1) Memory loss and convulsion are probably not necessary to produce clinical improvement. (2) Stimulation of cortical autonomic centers seems to be the most important factor in producing clinical improvement in the affective disorders with electroshock.

15651

Diabetes Mellitus in Rabbits Injected with Dialuric Acid.*

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(Introduced by Elliott P. Joslin.)

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Since the discovery that alloxan produces diabetes when injected into animals[1-3] other chemically related substances have been studied for possible diabetogenic action. Until recently no chemical with such action has been found.

Koref, Vargos, Rodriguez and Telchi[4] reported the production of diabetes with alloxantin and confirmation of this work has already been published from this laboratory.[5] Recently, Bruckman and Wertheimer[6] have found that besides alloxantin methylalloxan, dialuric acid, methyl dialuric acid and dimethyl alloxantin produce diabetes when injected into rats. The present investigation reports the production of diabetes in the rabbit with dialuric acid and compares the diabetes so produced, clinically and histologically, with the diabetes produced with alloxan.

Experimental. Eight chinchilla male rabbits each weighing 1500-2000 g were injected intravenously with dialuric acid in doses of 150, 200 or 250 mg per kg. The rabbits were not fasted at any time during the experimental period and in all but one instance the injection was made with the animal under nembutal anesthesia. Anesthesia seemed advisable since the dialuric acid was injected as a hot solution, owing to its insolubility at lower temperatures.

The solution of dialuric acid was prepared as a 1.5% solution in nearly boiling distilled water. The solution was cooled to a temperature of 40°-50° C and injected immediately. It was found necessary to keep the solution at this temperature to avoid recrystallization of the dialuric acid which tended to occur at room temperature.

Blood samples were taken from the ear veins before and at frequent intervals following the injection of the dialuric acid.

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* This investigation has been aided by a grant from the American Cyanamid Company, who also supplied the dialuric acid.

[1] Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

[2] Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

[3] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

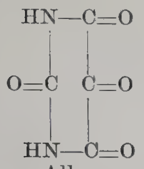
[4] Koref, O., Vargos, L., Rodriquez, F. H., and Telchi, A., *Endocrinology*, 1944, **35**, 391.

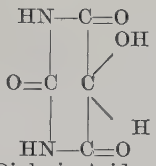
[5] Bailey, C. C., Bailey, O. T., and Leech, R. S., *Bull. New Eng. Med. Center*, 1945, **7**, 59.

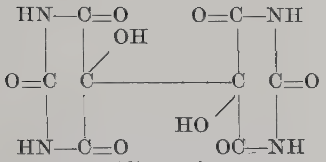
[6] Bruckmann, G., and Wertheimer, E., *Nature*, 1945, **155**, 267.

Determinations of blood sugar were made routinely by the micro procedure of Folin and Malmros[7] and in one instance (Rabbit No. 1) checked by a micro copper method referred to in an earlier paper.[8]

The chemical relationship between alloxan, dialuric acid and alloxantin is shown in the formulas below:


Alloxan


Dialuric Acid


Alloxantin

Dialuric acid is a reduction product of alloxan and may be oxidized to alloxan. That oxidation of dialuric acid to alloxan occurs easily in *very dilute solutions* in the presence of air has been emphasized by Archibald.[9] However, by applying Archibald's differentiating phosphotungstic acid test[9] to a warm 1.5% solution of dialuric acid (the concentration used in the experimental work) we have found less than 10% oxidation to alloxan occurring during the first 15 minutes at room temperature and only about 20% during a half hour at room temperature and reheating.

These results indicate that a solution of dialuric acid prepared as indicated contains during the first 30 minutes after its preparation at least 80% dialuric acid and probably no more than 10-20% alloxan.

Results. Chemical. The blood sugar results of the animals injected with 250 or 200 mg/kg are shown in Fig. 1. Three of these rabbits developed severe diabetes, one (No. 3) developed an initial blood sugar curve similar to the alloxan-injected animal but died 12 hours after the injection. Rabbit No. 5, though showing an initial hyperglycemia, did not develop diabetes.

Rabbits No. 6, No. 7 and No. 8, injected with 150 mg/kg, did not develop diabetes. No. 8 showed a tendency toward the transitory type of diabetes with one blood sugar of 278 mg % on the third day following injection, but a glucose tolerance on this rabbit 40 days after the injection was normal.

These results suggest that the rabbit requires a slightly larger dose of dialuric acid to produce permanent diabetes than of alloxan. If dialuric acid is given in 200-250 mg/kg doses, however, the blood sugar changes produced seem to parallel those observed in alloxan-injected animals.

Histology. Pancreas. The early stages of the pancreatic lesion were not studied since our purpose was to compare the fully developed lesions due to dialuric acid and to alloxan. The pancreas of the rabbits rendered diabetic by the injection of dialuric acid was characterized by paucity of islets and the small size of those remaining. A large percentage of the islets had disappeared completely. Their former sites were identified only with difficulty, being marked only by small masses of connective tissue without inflammatory cellular infiltration or residual traces of islet cells. In those islets which remained, the beta cells had entirely disappeared without concomitant infiltration with lymphocytes or polymorphonuclear leukocytes. Occasional alpha cells of normal morphology were seen, but more numerous alpha cells had pyknotic nuclei and agranular cytoplasm at the stage studied. The acinar and ductile tissues, stroma and blood vessels of the pancreas were within normal limits. The lesion was thus extremely similar to that seen in the pancreas in alloxan diabetes,[10] the only distinction being that some degenerating islet cells were present even when the rabbits were well established in diabetes.

Kidneys. There was considerable damage

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[7] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

[8] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

[9] Archibald, R. M., *J. Biol. Chem.*, 1945, **158**, 347.

[10] Bailey, O. T., Bailey, C. C., and Hagen, W. H., *Am. J. Med. Sci.*, 1944, **208**, 450.

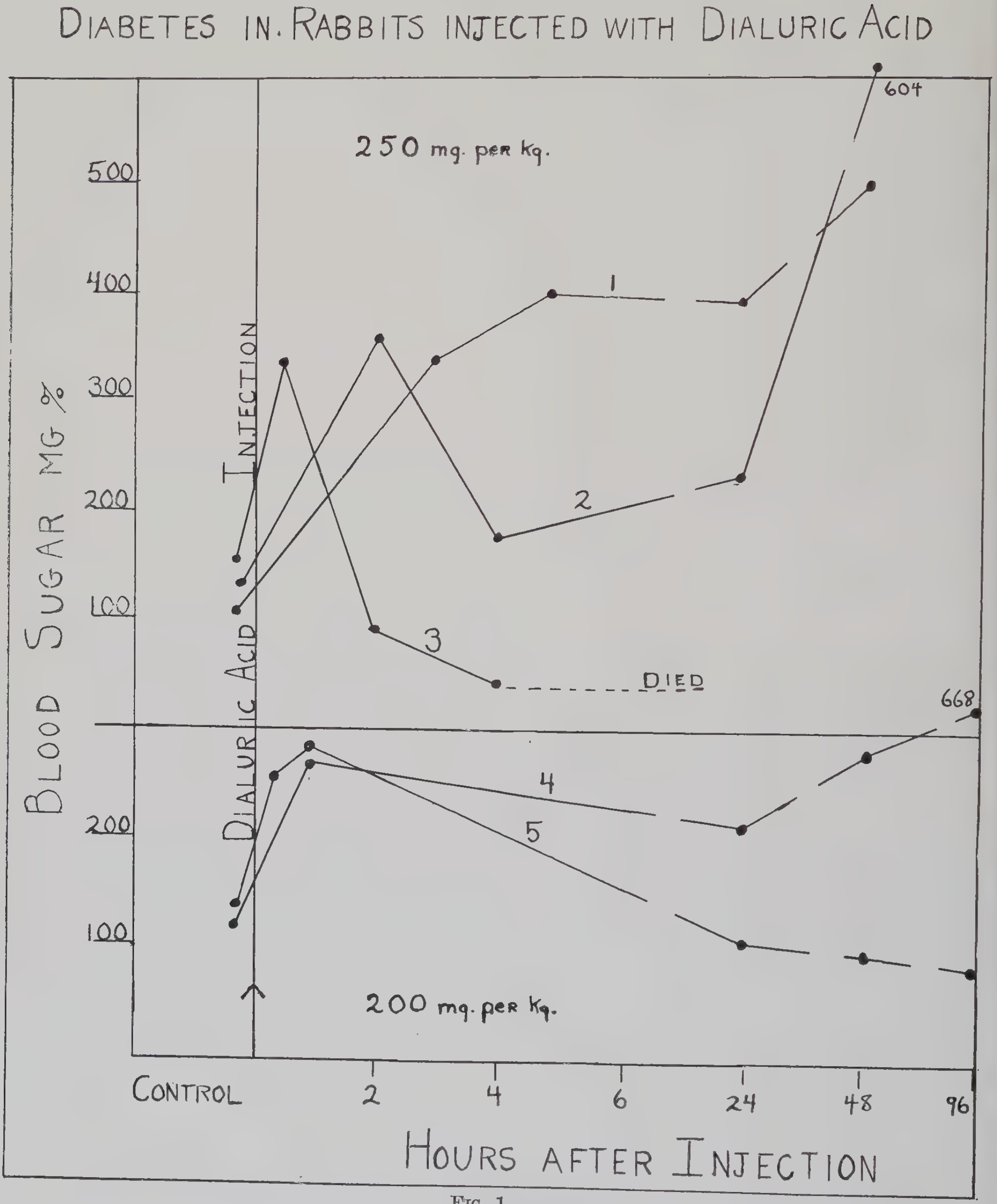

FIG. 1.

to the renal convoluted tubules. The tubular epithelia cells were swollen and the nuclei of many of them were pyknotic. They contained protein precipitate. The glomeruli, convoluted tubules, pelvic epithelium and blood vessels were within normal limits. The character of the renal damage was similar to that seen in alloxan diabetes. Since the extent of the kidney changes varied considerably in our large series of rabbits with alloxan diabetes, it is difficult to compare the degree of renal injury resulting from the injection of the 2 substances. In the material at hand, the renal changes due to dialuric acid seemed to be at least as great and possibly greater than those produced by alloxan.

Other Organs. The liver was somewhat congested, but there was no fatty infiltration. Other organs contained no lesions not found in control animals.

The adrenal showed no infiltration with polymorphonuclear leukocytes or other pathologic changes.

In Rabbit No. 3, which developed severe hypoglycemia and died during the night after injection of dialuric acid, the lesions were at an earlier stage than those of the diabetic animals. In many islets the central beta cells were pyknotic and the cytoplasm agranular, while some alpha cells at the periphery were essentially normal. In other islets all the cells had pyknotic nuclei. Again the degeneration and disintegration of the islet cells were unaccompanied by infiltration of inflammatory cells. The histologic features of other organs were similar to those already described in the diabetic animals.

The rabbit which developed transitory diabetes (No. 8) showed much less marked changes in the pancreatic islets. The alpha cells and many of the beta cells were normal in appearance. Some of the beta cells were pyknotic, while others were judged to have disappeared, since they were less numerous than in control rabbits. There was no inflammatory cellular infiltration. No hydroptic degeneration or mitoses were seen in the islet cells. The epithelial cells of the convoluted tubules of the kidney were swollen but there was less pyknosis and less protein precipitate in the lumen than in the diabetic animals.

In the rabbits (No. 5, 6, 7) not developing diabetes, either permanent or transitory, there were only a few pyknotic beta cells in some of the pancreatic islets and the other pancreatic changes were absent. The degree of renal damage, however, was as great as in the rabbits developing transitory diabetes.

These studies have been based on tissues fixed in acetic Zenker's solution and stained with phloxine-methylene blue and with Gomori's method for the cells of the pancreatic islets.

Discussion. Clinically the diabetes produced in rabbits by dialuric acid and by alloxan was identical except for the fact that a slightly larger dose is necessary when employing dialuric acid.

The triphasic blood sugar curve consisting of an initial transitory hyperglycemia followed by hypoglycemia and finally a permanent hyperglycemia or diabetes is found after the injection of either dialuric or alloxan. Permanent diabetes may be produced with either drug and with the use of smaller doses transitory diabetes may occur.

The histologic changes described as the result of injection of dialuric acid are closely similar to those due to the injection of alloxan. The few variations mentioned are trifling when considered from the functional point of view. The cardinal features of alloxan diabetes in the rabbit—necrosis of islet cells with survival of a few alpha cells, moderate to mild injury to the renal convoluted tubules, with only minor changes in other organs—are duplicated in dialuric acid diabetes. Such evidence as can be gleaned from these experiments suggests that dialuric acid diabetes, like alloxan diabetes, is due to a lesion of the islets of Langerhans, which is degenerative from its inception.

Since dialuric acid may be obtained as a reduction product of alloxan, it might be questioned whether one of these substances is converted into the other in the body of the experimental animal before the lesions are initiated. The present series of experiments throws no light on this question other than to indicate that the lesions caused by the 2 substances are similar and are consistent with either view.

Dialuric acid is not recommended as a substitute for alloxan as an experimental technic for the production of diabetes, because it must be injected in a warm solution owing to its low solubility. Except for the extra labor involved, however, dialuric acid and alloxan may be regarded as interchangeable as diabetogenic agents.

Summary. Dialuric acid when injected intravenously into rabbits produces diabetes which is indistinguishable clinically or pathologically from alloxan diabetes.

15652

Action of Urea and Some of Its Derivatives on Bacteria. V. Antibacterial Activity of Methyl- and Thiourea.*

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It has been reported[1-3] that urea and urethane are highly bacteriostatic and bactericidal for many Gram-negative and several Gram-positive organisms, potentiate moderately the activity of sulfonamides, inhibit *p*-aminobenzoic acid moderately, and increase the solubility of sulfanilamide and sulfathiazole. To determine whether increased bacteriostatic potency results from substitution of one of the NH_2 groups in urea by $—CH_3$ radicals, as was suggested by the superiority of urethane over urea, other derivatives of carbamide were investigated. The present paper deals with a study of antibacterial and anti-*p*-aminobenzoic acid activity of methyl and thio derivatives of urea, the $—CH_3$ group replacing one $—NH_2$ in the first compound and —S being substituted for the =O in urea in the second.

A review of the literature reveals no information concerning the activity of methyl urea against microorganisms. Thiourea is reported[4] to be antibacterial and to increase the effectiveness of sulfathiazole. This action was thought to be synergistic.

Methods. The methods used in the studies described in this paper are similar to those detailed in the previous ones of this series and, therefore, will not be described here. Veal-infusion broth with added horse serum is designated as VIB-S. The pH of 5% methyl- and thiourea solutions in VIB-S was found to be 7.2 to 7.25.

Results. I. *The Bacteriostatic Effects of Methyl Urea.* A study of the bacteriostatic effect of methyl urea in amounts varying from 2 to 8% in VIB-S revealed that a concentration of approximately 6% produced suppression of growth of several Gram-negative organisms. *Ps. aeruginosa* was completely inhibited by a 4% solution of the drug. Moderate bacteriostasis of *E. coli, S. schottmulleri, P. vulgaris,* and *E. typhi* resulted from exposure to 5% methyl urea. *P. vulgaris* was partially inhibited by 6% and an 8% solution completely arrested growth for 96 hours.

In a synthetic medium,[5] the concentration

TABLE I.
Effect of Varying Concentrations of Methyl Urea on Growth of Various Organisms in Different Media.

			Concentration of methyl carbamate Growth at various periods											
			6%		5%		4%		3%		2%		No drug	
Medium	Organisms inoculated	hrs	48	96	48	96	48	96	48	96	48	96	48	96
VIB-S	*E. coli*—6500		0	0	0	3+	2+	4+	4+	4+	4+	4+	4+	4+
	S. schottmulleri—1050		0	0	0	2+	1+	3+	4+	4+	4+	4+	4+	4+
	Ps. aeruginosa—29,000		0	0	0	0	0	0	1+	3+	4+	4+	4+	4+
	*P. vulgaris**—34,000		0	2+	1+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	E. typhi—525		0	0	0	0	0	0	1+	3+	3+	4+	4+	4+
Synthetic medium	*E. coli*—22,000,000		0	0	0	0	0	0	0	3+	4+	4+	4+	4+

* 8% completely inhibited for 96 hours.

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* This study was aided by a grant from the Johnson Research Foundation, New Brunswick, N.J.

[1] Weinstein, L., and McDonald, A., *Science*, 1945, **101**, 44.

[2] Weinstein, L., and McDonald, A., *J. Immunol.*, 1946, **54**, 117.

[3] Weinstein, L., and McDonald, A., *J. Immunol.*, 1946, **54**, 131.

[4] Lee, S. W., Epstein, J. A., and Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 105.

[5] Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

TABLE II.
Effect of Two Concentrations of Methyl Urea on Varying Amounts of Para-Aminobenzoic Acid in VIB-S.
Inoculum of *E. coli*—810,000 organisms.

Conc. of methyl urea, %	Sulfanilamide mg/100 ml	*p*-aminobenzoic acid mg/100 ml	Growth (hr) 24	48	72	96
2	—	—	4+	4+	4+	4+
4	—	—	4+	4+	4+	4+
—	15	0.001	0	0	0	3+
—	15	0.0025	0	0	3+	4+
—	15	0.005	0	3+	4+	4+
—	15	0.0075	0	4+	4+	4+
—	15	0.01	2+	4+	4+	4+
—	15	0.025	4+	4+	4+	4+
—	15	0.05	4+	4+	4+	4+
—	15	1.00	4+	4+	4+	4+
—	15	2.50	4+	4+	4+	4+
2	15	0.001	0	0	0	0
2	15	0.0025	0	1+	4+	4+
2	15	0.005	0	3+	4+	4+
2	15	0.0075	1+	4+	4+	4+
2	15	0.01	2+	4+	4+	4+
2	15	0.025	3+	4+	4+	4+
2	15	0.05	4+	4+	4+	4+
2	15	2.50	4+	4+	4+	4+
4	15	0.001	0	0	0	1+
4	15	0.0025	0	0	0	2+
4	15	0.005	0	0	3+	4+
4	15	0.0075	0	1+	4+	4+
4	15	0.01	0	2+	4+	4+
4	15	0.025	0	2+	4+	4+
4	15	0.05	0	2+	4+	4+
4	15	0.75	0	2+	4+	4+
4	15	0.10	0	3+	4+	4+
4	15	2.50	0	3+	4+	4+
—	—	2.50	4+	4+	4+	4+
2	15	—	0	0	0	0
4	15	—	0	0	0	0
—	15	—	0	0	0	0
—	—	—	4+	4+	4+	4+

TABLE III.
Bacteriostatic Effect of Thiourea in Serum-Veal-Infusion Medium.

		Concentration of thiourea. Growth at various periods									
		None		2%		1%		0.5%		0.25%	
Organisms. No. in inoculum	hrs	48	96	48	96	48	96	48	96	48	96
E. coli—3250		4+	4+	0	0	0	0	1+	1+	3+	4+
S. aureus—2400		4+	4+	0	0	0	1+	1+	3+	4+	4+

of methyl urea required to suppress growth of *E. coli* was found to be less than that necessary in VIB-S.

A study of the potentiating effect of methyl urea on sulfanilamide in a synthetic medium, using *E. coli* as the test organism, revealed that a 2% concentration of the carbamate produced no apparent increase in activity of the sulfonamide. Four per cent methyl urea was bacteriostatic when used alone and any stimulation of the activity of sulfanilamide by it could, therefore, not be determined.

II. *The Effect of Methyl Urea on Para-aminobenzoic Acid.* A 2% concentration of methyl carbamate did not inactivate *p*-aminobenzoic acid. Four per cent was moderately effective, the sulfonamide-inhibiting substance being inactivated in amounts as large as 0.1 mg per 100 ml for 24 hours. As with urea and urethane, the anti-PABA activity of methyl urea appeared to be of short duration.

III. *The Bacteriostatic Effects of Thiourea.* A 2% concentration of thiourea inhibited growth of *S. aureus* for 96 hours, 1% for only 48 hours. Quantities less than 1% were ineffective. One per cent thiourea suppressed completely the multiplication of *E. coli.*

Summary and Discussion. Methyl urea was found to be bacteriostatic for several Gram-negative bacteria in both VIB-S and in a synthetic medium. The concentration of drug required to suppress growth varied somewhat with each organism, but it ranged generally between 6 and 8% except for *Ps. aeruginosa,* which was inhibited by a 4% solution. The activity of the drug in a synthetic medium was greater than in VIB-S.

The substitution of one $—NH_2$ in carbamide by a single $—CH_3$ group increased the activity of urea very little since approximately the same concentrations of the 2 compounds were required to inhibit the growth of bacteria. Urethane, as previously reported, was more highly bacteriostatic than either urea or methyl urea. It would appear, therefore, that the insertion of $—CH_2CH_3$ in place of one $—NH_2$ in urea results in greater activity than that following the addition of only a single carbon atom. As shown by the few data presented, the replacement of $=O$ by $—S$ in the urea molecule is apparently very effective in increasing its antibacterial properties since a 1% concentration of thiourea produced approximately the same results with *E. coli* as 3% urethane or 6% urea.

Conclusions. 1. Methyl- and thiourea are bacteriostatic for Gram-negative bacteria. 2. The substitution of an $—NH_2$ group by $—CH_3$ in urea results in no demonstrable increase in antibacterial activity. 3. The replacement of the $=O$ group in urea by $—S$ increases markedly the antibacterial activity. 4. Methyl urea, in the concentrations studied, does not potentiate the sulfonamides. It does have a moderate antagonistic effect on *p*-aminobenzoic acid.

15653

Interrelation Between α-Tocopherol and Protein Metabolism: Body Weight and Tooth Pigmentation of Rats.*

E. L. HOVE. (Introduced by Philip L. Harris.)

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A relation between vitamin E and protein metabolism has been suggested in 3 reports in the literature. Cerecedo and Vinson[1] observed that a 58% litter incidence of weanling paralysis in mice was reduced to 28% by increasing the casein in the diet of the mothers.

Dam[2] noted that when rats were placed upon a low-protein diet, the group receiving α-tocopherol survived somewhat longer than the E-free controls. However, the *rate* of body weight loss was the same in the 2 groups, and the E-fed group had a lower body weight at death. Victor and Pappenheimer,[3] on the other hand, reported a very marked effect of tocopherol in conserving the body weight of rats on a low-casein diet. This work is complicated by the fact that a cirrhosis-producing diet (low choline as well as low casein) was used. The effect of tocopherol possibly could have been related to the altered metabolism in the cirrhotic state.

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* Communication No. 106.

[1] Cerecedo, L. R., and Vinson, L. J., *Fed. Proc.*, 1944, **3**, 55.

[2] Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 55.

Since these 2 reports appear to be contradictory in some respects, we have repeated the work of Victor and Pappenheimer using equalized feeding of a diet low in casein but otherwise nutritionally adequate.

Fifty-four male weanling rats from the stock colony were placed upon a vitamin E-free diet of the following composition: crude casein, 22.5; sucrose, 63; salt mixture USP No. 2, 4.5; and lard, 10. B-complex vitamins were added to the casein to furnish 10 γ of thiamine, riboflavin and pyridoxine, 25 γ calcium pantothenate, 100 γ niacin, 1 mg of choline chloride, 0.1 mg *i*-inositol, and 5 γ vitamin K per g of ration. Each rat was supplemented weekly with 2,000 units vitamin A and 20 units of vitamin D in olive oil solution.

The animals were kept on this diet, *ad libitum,* until they reached 225 g in body weight. This required 5 to 7 weeks. As they attained this weight the rats were placed on a fixed daily amount of one of 3 diets which differed only in their casein content. These diets contained 5, 20, or 40% crude casein, and, except for compensatory changes in sucrose, were identical in composition with the diet described above.

A daily allotment of 8.9 g of the diet was fed to each rat in the morning and was generally consumed within an hour. The diets were made fresh weekly and kept refrigerated at all times. Half of the rats on each diet were supplemented with daily doses of 1 mg of d, α-tocopherol in olive oil solution.

The average body weight changes of the rats on the 5% casein diet (8.9 g daily) are shown in Fig. 1. These animals lost about 40 g in body weight during the first 4 weeks, after which the curves levelled off. α-Tocopherol had little influence during this period of adjustment to the new diet. Beginning at about the 10th week the vitamin E-low rats began a rapid weight loss. They continued to consume their daily allotment of food in spite of declining body weight. Their general appearance deteriorated rapidly. They became listless and showed marked signs of atony and muscular dystrophy. During the last 3 weeks on experiment 3 of the 9 animals regularly left up to 3 g of their diet unconsumed.

In sharp contrast to the vitamin E-low group, the animals receiving α-tocopherol regained some of their initial weight loss and thereafter maintained their weight. In spite of the low-protein intake their appearance was good. They were alert and showed normal general muscular tone. Statistically the difference in body weight between the E-low and E-fed groups was highly significant. At 21 weeks on experiment the standard error for the average weight of both groups was ±3.5; this corresponds with a *t* value of

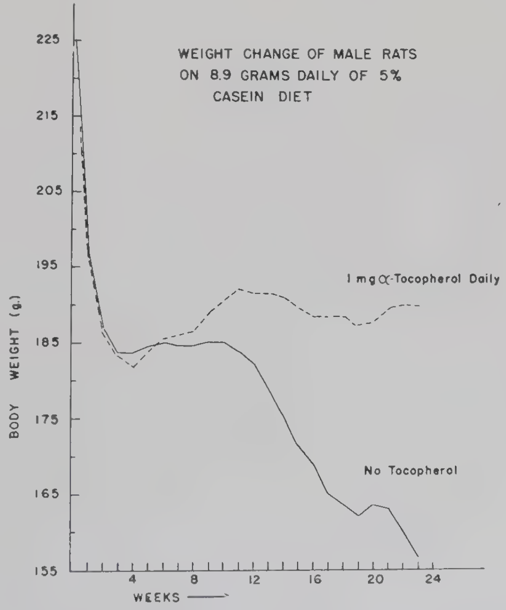

FIG. 1.

The effect of α-tocopherol on body weight changes of adult male rats shifted to a 5% crude casein diet.

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[3] Victor, J., and Pappenheimer, A. M., *J. Exp. Med.*, 1945, **82**, 375.

6.2, while for P (0.01) the theoretical t value is 2.95.

At autopsy no signs of liver cirrhosis were evident in either group. The gross appearance and fat content of the livers were normal. Very marked atrophy of the testes was seen in the E-free group; their average testes weight was 0.4 g as compared with an average of 1.2 g in the E-fed group. Eight of the 9 low-E rats had marked hyperplastic crater lesions in the rumen of the stomach, while only 3 of the E-fed rats showed such lesions, and these were mild.

The animals on the higher protein diets required about 2 weeks to adjust themselves to the fixed limited quantity of the new diets. They lost weight during this period. However, after this time they resumed their growth. At the end of 24 weeks the group on the 20% casein diet averaged 292 g while the corresponding E-fed group averaged 303 g. On the 40% casein diet, the average weights were 298 and 308 g for the E-low and E-fed groups respectively. The animals all appeared normal. No signs of dystrophy were yet evident.

An interesting incidental observation has been made on tooth pigmentation in these rats. Granados and Dam[4] have shown that a combination of high-fat and low-E leads to loss of the normal tooth pigment. Our experiment has added protein to the factors influencing this yellow-orange color of the teeth. Table I shows the average color index of the maxillary incisors of the various groups after 10 and 20 weeks on the fixed daily intake of the diets. The color index was rated on an arbitrary scale with 5 degrees ranging from "0" to "4." All of the tocopherol-fed animals had essentially normal tooth color, regardless of protein content of the diet. However, it is clear that the amount of casein greatly influenced the tooth color in the non-tocopherol groups.

TABLE I.
Effects of α-Tocopherol and Protein on Tooth Pigmentation of Adult Rats Fed 8.9 g of Diet Daily. The values represent the average color ratings (0 to 4) of the groups.

Casein content of diet (%)	No tocopherol		Plus tocopherol	
	10th wk	20th wk	10th wk	20th wk
5	0.1	0	3.2	2.8
20	1.4	0.9	3.3	3.2
40	1.6	1.1	3.6	3.2

Discussion. Under the conditions of this experiment α-tocopherol was essential to the maintenance of body weight and general well-being of adult rats on a low-casein diet. The daily food intake was fixed at a level which, with a normal protein percentage, allowed continued growth and well-being even without tocopherol.

This may indicate that α-tocopherol "spares" or allows better utilization of some constituent of the casein. Conversely, the results may mean that low casein (or some constituent) greatly increases the requirement of the rat for vitamin E. The second explanation appears the more plausible and is currently being checked with rabbits as the experimental animal. Several pure amino acids have been added to low-casein diets at a 1% level in an attempt to find some specific factor which would counteract the dystrophy-inducing property of the low-casein diet. No benefit has been observed with tryptophane, valine, arginine, or lysine. A questionable benefit was seen with *l*-cystine. This work is being continued.

In the work of Dam[2] tocopherol apparently had no influence on the loss in body weight of rats on diets in which the only protein was that furnished by 10% yeast. However, in our work as well as that of Victor and Pappenheimer[3] tocopherol had a marked influence when the only protein was 5% crude casein. The difference in composition of casein and yeast protein may, therefore, furnish a clue as to a specific factor involved in vitamin E metabolism.

Summary. Adult male rats have been fed fixed daily quantities of vitamin E-low diets containing 5, 20, or 40% crude casein. On the 5% casein diet the animals lost weight and developed muscle dystrophy, testicular atrophy, stomach ulcers, and tooth depigmentation.

These symptoms of a vitamin E deficiency were prevented either by α-tocopherol or by the higher protein diets. The possible significance of a lack of individual amino acids in increasing the vitamin E requirement of rats is discussed.

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[4] Granados, H., and Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 295.

15654

Induced Ovipositions in Relation to Age of Oviducal Egg in the Domestic Hen.

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The removal of the ruptured follicle from the ovary of the hen results in a delay in the time of lay of the egg which arose from that follicle.[1] The experiments reported here are an approach to the problem of how the ruptured follicle functions to determine the time of lay. Specifically, they were designed to determine whether the sensitivity of the chickens' uterus to an oxytocic agent increases as the time of normally expected lay approaches.

Methods and Materials. Five groups of laying hens were injected intravenously with the same dose of an oxytocic agent at various hours after the ovulation of an egg. The results were expressed as the percentage of birds showing premature ovipositions.

Birds selected for injection carried in their oviducts the second egg of a 2-egg clutch, the first egg of which was laid at the recorded hour of either 10:00 a. m. or 11:00 a. m. The time of lay of all eggs in this laboratory is recorded on the hour every hour between 8:00 a. m. and 4:00 p. m., so that there was a maximum spread of no more than 2 hours between the time of lay of the earliest and latest first egg of a clutch in any group. Of all the birds used, those selected for 10:00 a. m. lay of the first egg of a clutch comprised between 55 and 60% of the total group in 4 groups and 74% of the total in the fifth group.

The presence of the oviducal egg was determined by digital palpation through the rectum. The age of the oviducal egg at the time of injection was estimated from the relationships between time of lay and ovulation of intraclutch eggs,[2,3] and the time required for the egg to pass through the various portions of the oviduct.[2,3] For 2-egg clutches, the interval between lay of the first egg and ovulation of the second is about 45 minutes,[2,3] and the time required for the egg to reach the uterus, about 4 hours from the time it enters the oviduct.[2]

The injections were made during the interval between ovulation and the time of expected lay (a period of about 27 hours) at the following hours: 4:00 p. m., 8:00 p. m., midnight, 6:00 a. m. and 10:00 a. m. Usually, no more than one minute elapsed between the injection of one bird and the injection of the next in each group; the maximum difference between the given times of injection and the time of the earliest or latest injection in the group was 34 minutes. White Leghorn and Rhode Island Red hens were used: the number of one breed did not exceed the number of the other by more than one bird in each group, except in that injected at 6:00 a. m. In this group there were 33 White Leghorns and 12 Rhode Island Reds. The results for the groups as a whole were not appreciably different from the results obtained in each breed.

The agent used to induce premature oviposition was Parke, Davis & Co. "Pitressin." Although the main constituent of this preparation is the pressor principle of the posterior

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[1] Rothchild, I., and Fraps, R. M., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 79.

[2] Phillips, R. E., and Warren, D. C., *J. Exp. Zool.*, 1937, **76**, 117.

[3] Unpublished data from this laboratory.

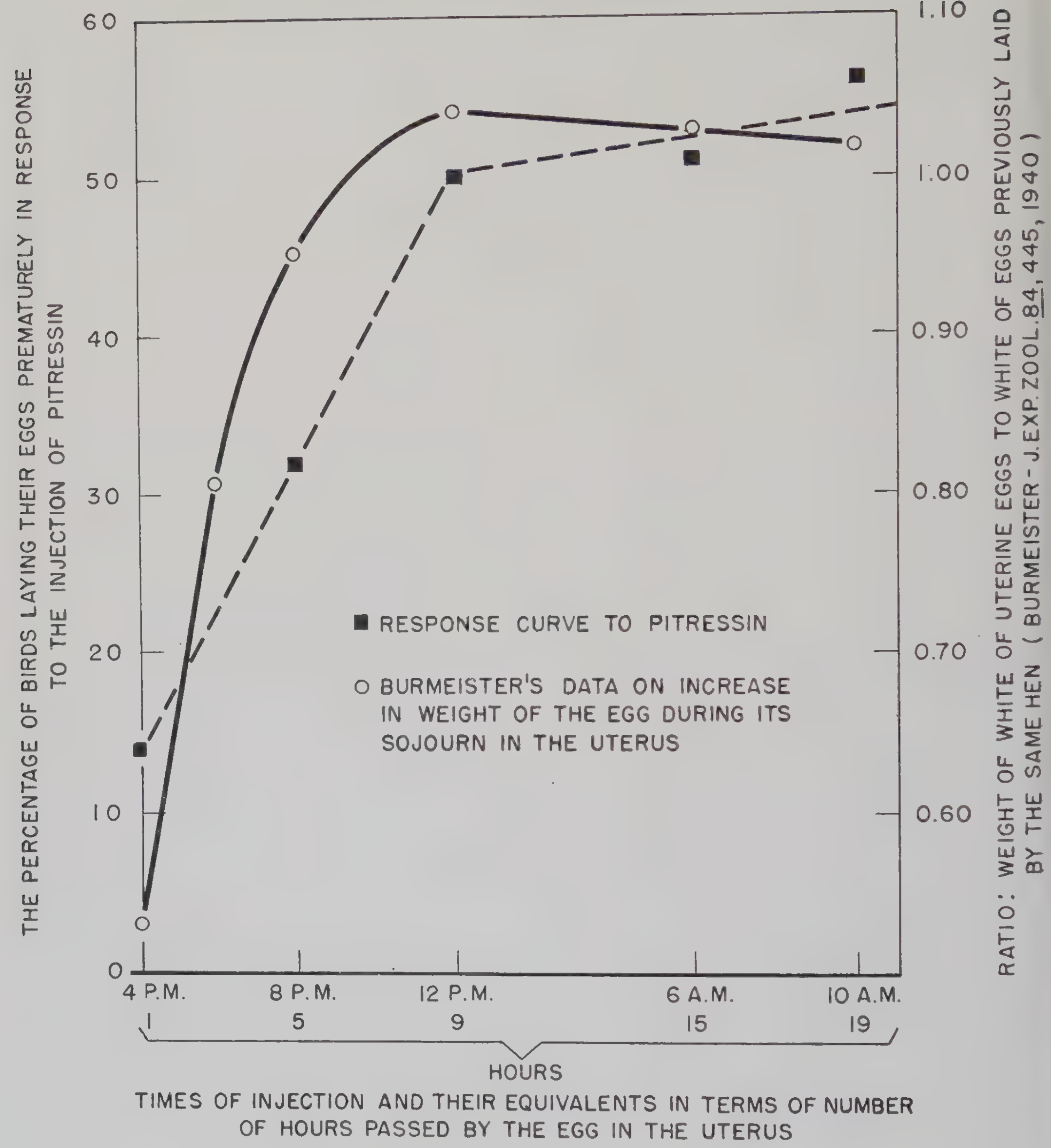

FIG. 1.

Comparison of the curve showing percentage response to Pitressin in relation to the age of the oviducal egg with the curve relating increase in size of the egg to the length of time spent by the egg in the uterus.

pituitary, it has been shown by Burrows and Fraps[4] to have a strong oxytocic action in the chicken. The dose used was 0.30 unit of labeled potency; this was prepared by diluting 1.0 cc, taken from a separate 1.0 cc vial, and containing 20.0 pressor units, to 10.0 cc with 0.9% NaCl. The amount injected was 0.15 cc. A diluted preparation was never used for more than 2 days in succession. The same 0.5 cc hypodermic syringe was used for all injections.

A premature oviposition in response to Pitressin was taken as lay of the oviducal

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[4] Burrows, W. H., and Fraps, R. M., *Endocrinology*, 1942, **30**, 702.

TABLE I.
Relation Between the Percentage of Birds Reacting by Premature Oviposition to a Constant Dose of Pitressin (0.30 Unit) and the Hours After Ovulation That the Injection Was Made.

Injections		Birds injected		
			Positive reactions*	
Time	Approx. No. hr after ovulation	No.	No.	%
4 p.m.	5	22	3	14
8 ”	9	31	10	32
12 ”	13	44	22	50
6 a.m.	19	45	23	51
10 ”	23	32	18	56

* A positive reaction is the expulsion of the oviducal egg in response to the injection of Pitressin within 30 minutes after injection.

egg within a period of 30 minutes from injection. Practically all premature ovipositions, however, occurred well within 15 minutes from the time of injection.

Results are shown in Table I. Between 4:00 p. m. and midnight following the ovulation of these particular eggs, the percentage of premature ovipositions in response to 0.30 unit of Pitressin increased substantially. From midnight to 10:00 a. m. the next day, however, there was only a slight, if at all significant further increase in the percentage of premature ovipositions. Since 10:00 a. m. was only 2 to 6 hours from the time of normally expected lay for these particular eggs, it can probably safely be assumed that there was very little change in the response to a given dose of Pitressin from midnight until the actual time of lay.

Removal of the ruptured follicle apparently did not affect the percentage of birds responding by premature oviposition to this dose of Pitressin. Of 17 birds so treated, 9, or 53%, laid prematurely. This compares very well with the group of intact birds injected at the same hour. This fact, together with the evidence of a plateau from midnight on, indicated that the response curve might be a manifestation of the mechanical stretching of the uterine walls as the egg increases in size (plumping) with the uptake of water.[5] To check this, Burmeister's data[5] on the increase in weight of the egg in relation to time spent in the uterus was compared with the response curve to Pitressin. The 2 curves are shown in Fig. 1. For comparison, the hour of injection was made equivalent to time spent in the uterus by means of the following rough calculations. The hour of lay of the first egg of the clutch for each group as a whole was taken as 10:00 a. m. (This probably errs somewhat in the direction of a later-than-actual average, but not enough to affect appreciably the overall picture). Ovulation of the test egg would therefore have occurred about 10:45 a. m., and the egg would have entered the uterus somewhere around 2:45 p. m.-3:00 p. m. (see above). The 2 curves were plotted, taking 3:00 p. m. as the 0 point for time spent in the uterus. The response curve shows the beginning of a plateau very close to the time that the egg becomes fully plumped, *i.e.*, reaches its almost maximum size. The only further increase in size that takes place from this point on is that due to shell formation (which is not shown by Burmeister's[5] figures), and it may well be that the slight increase in the percentage response to Pitressin during the period of shell formation is a reflection of this phenomenon.

Discussion. The increasing percentage of birds responding to a given dose of Pitressin as their eggs increase in size, and the lack of any substantial further increase in response after the egg reaches its almost maximum size, may be taken to indicate that the changes in reactivity of the uterus during the sojourn in it of the egg are not directly responsible for the determination of the time of normal oviposition. With the reservation that a response to Pitressin is only a probable indication of the sensitivity of the uterus to the stimulus which under normal conditions leads to oviposition, it may be assumed for the present, therefore, that the probable mode of action of the ruptured follicle is one that primarily involves a change in some system external to the uterus.

Summary. Hens carrying oviducal eggs were injected with 0.30 unit of Pitressin at times equivalent to 1, 5, 9, 15, and 19 hours of time passed by the egg in the uterus. The percentages of birds laying prematurely were respectively 14, 32, 50, 51, and 56.

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[5] Burmeister, B. R., *J. Exp. Zool.*, 1940, **84**, 445.

Removal of the ruptured follicle shortly after ovulation did not alter the response at the 19th hour in the uterus stage. The beginning of the plateau of the response curve approximately coincided with the time at which the oviducal egg became fully plumped, *i.e.,* reached its almost maximum size. The response curve was interpreted as the expression of a change in the mechanical relations between the uterus and the contained egg, rather than a change in intrinsic sensitivity due to the action of the ruptured follicle.

15655

Physiological Disposition of Penicillin G and K in Dogs.

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The unexpected low activity of penicillin K in experimental syphilis first observed by Chesney[1] has been confirmed.[2] In addition it has been demonstrated that penicillin K is less effective than G in the treatment of mice infected with pneumococcus Type I,[3] *Streptococcus pyogenes*[3] and *Borrelia novyi.*[4,5]

This low activity of penicillin K has been correlated with an apparently low plasma concentration and it has been suggested that this is a result of more rapid destruction in the animal body.[3,6] A more detailed study of the disposition of penicillin G and K in dogs has led us to a somewhat different conclusion, and has cast considerable doubt on the supposition that inactivation plays a major part in the immediate fate of injected penicillin K.

Recovery of Added Penicillin G and K from Body Fluids and Tissues. The recovery of added penicillin from various biological materials was studied by the customary cup test procedure of assay for penicillin concentration. Known amounts of each penicillin* were added to body fluids and to tissue mashes, and the actual assay figures in each case were related to the assay figures of the same concentration of penicillin added to saline.

Plasma was obtained by centrifuging oxalated or heparinized dog blood. Tissue mashes were prepared with a homogenizer, using 2 parts of saline for one of muscle, and equal parts of saline and tissue in all other cases. The grinder was immersed in ice water during the preparation of each tissue. One-tenth of a volume of an appropriate saline solution of penicillin was added to a series of samples of tissue mashes, plasma, and saline. Final concentrations of penicillin ranged from 0.3 μg per ml to 24 μg per

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[1] Unpublished observations quoted by Eagle and Musselman, *Science,* 1946, **103**, 618.

[2] Rake, G., Dunham, W., and Donovick, R., to be published.

[3] Eagle, H., and Musselman, A., *Science,* 1946, **103**, 618.

[4] Burk, M., Farr, A. C., and Schnitzer, R. J., *Science,* 1946, **104**, 370.

[5] Richardson, A. P., Loeb, P., and Walker, H. A., unpublished observations.

[6] Coghill, R. D., Osterberg, A. E., and Hazel, G. R., *Science,* 1946, **103**, 709.

* We are indebted to Drs. Wintersteiner and Adler of the Division of Organic Chemistry, and Mr. Lott and Mr. Dolliver of the Division of Medicinal Chemistry for these preparations. Elementary analysis and bioassay values for both penicillins agreed well with theoretical values. Analysis by Craig distribution methods showed the G preparation to be at least 92% homogeneous, the remainder consisting of inactive components. The K preparations were at least 88% of "K type" penicillins, not more than 10% of an active fraction which was probably an "F type" penicillin, and the remainder was an inactive component.

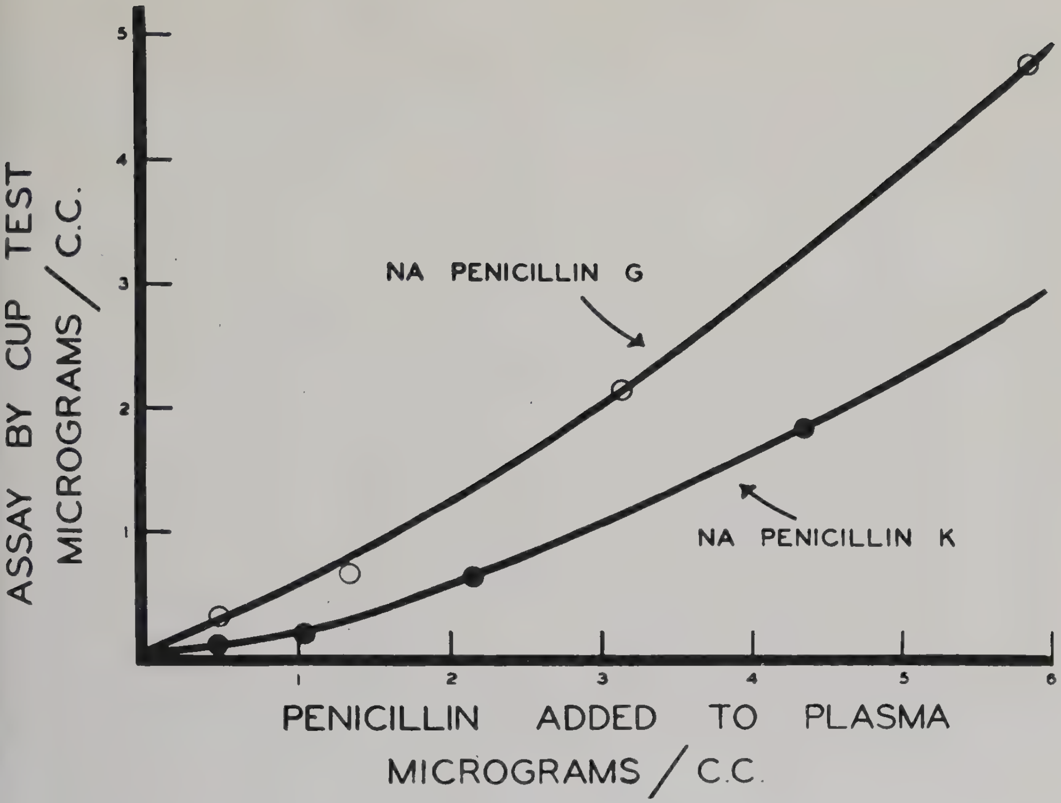

Fig. 1.
Recovery of Penicillin G and K from dog plasma, when assayed by the cup test procedure. Each curve represents an average of six experiments carried out on different days.

ml. The assays were then carried out by the usual cup test procedure.†

The recovery of penicillin added to saline was within 10% of the predicted concentration in all cases. With all tissues and plasma, the recoveries were incomplete and the extent of the loss was inversely proportional to the concentration of penicillin. Fig. 1 summarizes a portion of 6 typical experiments obtained with plasma, and is presented because it shows a marked difference in behavior of the 2 penicillins when added to this body fluid. It is quite obvious that cup test assays of penicillin K in plasma are subject to considerable error, unless a correction is made for incomplete recovery. Proof that the incomplete recoveries were due to the assay procedure used and not due to destruction of penicillin K was obtained by 2 experiments.

1. Simultaneous assay of representative samples of penicillin-plasma mixtures by the tube dilutions technics gave complete recoveries. For example, in one experiment in which 1.25 μg per ml of K was added to plasma, cup test assays gave 20% recovery, whereas tube dilutions assays gave 100%

TABLE I.
Average Per Cent Recovery of Penicillin G and K from Dog Plasma and Tissue Mash by Cup Test at Different Concentrations.

	5 γ/ml		1 γ/ml	
	G	K	G	K
Plasma	83.0	42.5	65.0	17.1
Liver	63.5	64.5	45.0	28.2
Kidney	62.5	55.5	35.0	25.0
Spleen	75.0	50.0	45.0	42.0
Brain	87.0	78.0	60.0	37.5
Muscle	73.0	80.0	60.0	54.0
Lung	54.0	66.0	45.0	44.0

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† Bioassays reported in this study were carried out under the general supervision of the Division of Microbiology.

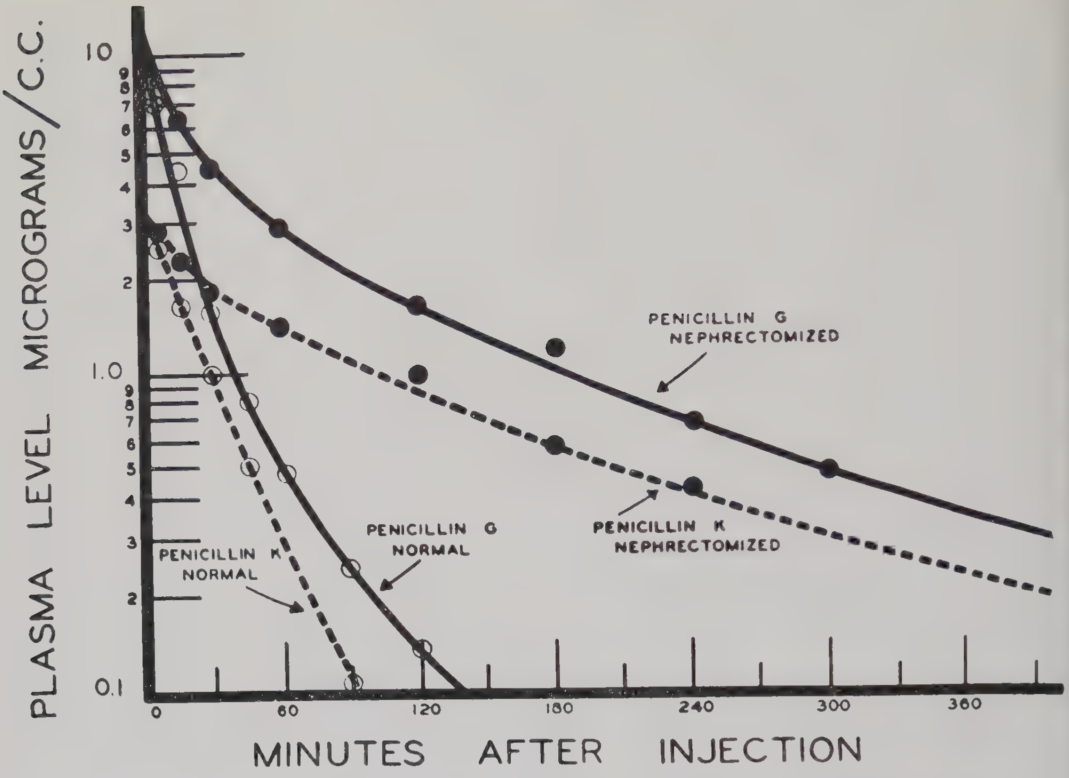

FIG. 2.
The rate of disappearance of penicillin G and K from the plasma of dogs following intravenous injection of 2 mg per kilo. Each curve is the mean of experiments in 6 dogs. All figures have been corrected for error in the cup test assay.

recovery.

2. Incubation of plasma-penicillin mixtures for 3 hours at 22°C or 24 hours at 4°C gave the same recovery as when the penicillin was added immediately preceding assay. Thus if destruction had been responsible for the low cup test assay it must have occurred immediately and then stopped within a few minutes.

The results of all recovery experiments with plasma and tissues are summarized in Table I. The per cent recovery for each material was obtained by plotting added penicillin against assay results in a form similar to Fig. 1. Per cent recovery of the 2 concentrations was determined from the curves thus obtained. All results reported later in this paper have been corrected by the use of such curves to give "true" penicillin concentration.

Rate of Disappearance of Penicillin G and K from the Plasma of Normal and Nephrectomized Dogs. The rate of disappearance of a drug from the plasma of an intact animal is the net result of distribution, excretion and destruction. In the case of penicillin it is well recognized that rate of disappearance is conditioned largely by renal excretion. Therefore, the rate of fall of plasma-penicillin in a nephrectomized dog should reflect distribution, destruction and extrarenal excretion.

Experiments were set up as follows: each dog was injected intravenously with 2 mg per kg of the appropriate penicillin. Blood samples were taken at short intervals of time until the plasma concentration approached zero. The following day a nephrectomy was performed, and after 24 hours recovery, the same dose of the same penicillin was injected intravenously and rate of disappearance from plasma again determined. Six

TABLE II.
Distribution of Penicillin G and K Between Plasma and Tissues of Dogs Following Continuous Intravenous Injection.

Dog No.	Plasma level, γ/ml		Plasma-tissue ratios after 2 hrs infusion						
	After 1 hr Infusion	After 2 hr Infusion	Cerebrospinal Fluid	Brain	Muscle	Spleen	Lung	Liver	Kidney
Penicillin G—0.1 mg/kilo/minute.									
25	13.8	23.5	151.0	47.0	5.2	5.9	2.1	3.3	0.25
26	15.0	24.7	185.0	62.0	5.1	9.5	2.0	2.7	1.1
27	11.2	16.0	191.0	32.0	4.4	3.8	1.4	3.6	0.42
28	17.3	14.6	91.0	18.4	6.1	6.1	2.1	1.4	0.43
29	24.3	28.1	220.0	56.0	5.9	4.5	2.7	2.7	0.64
30	15.1	16.7	230.0	69.0	10.6	5.2	2.4	1.9	0.46
Mean	16.1	20.6	176.0	47.4	6.2	5.8	2.1	2.4	.55
Penicillin K—0.2 mg/kilo/minute.									
31	12.5	18.3	62.0	18.3	4.7	4.6	2.7	1.8	0.55
32	17.5	24.8	220.0	20.6	8.3	9.6	4.8	1.9	0.91
33	13.6	13.5	151.0	34.0	6.1	9.1	3.2	1.1	0.52
34	12.6	13.5	135.0	22.5	6.4	8.5	3.8	1.2	0.81
35	19.5	27.0	270.0	45.0	*	*	*	1.2	0.82
Mean	15.1	19.4	167	28.1	6.4	7.9	3.8	1.4	0.72

* Not determined.

dogs were used for each penicillin. Scrutiny of the data (Fig. 2) shows a number of points of interest:

1. In as short a time as 5 minutes after injection the plasma level of penicillin K is less than one-half that seen when the same dose of G was injected. This finding suggests that K is either destroyed within 5 minutes or some other factor is responsible for the disposition of K as compared to G.

2. From a given plasma level the rate of disappearance of the 2 penicillins is not greatly different. If destruction had been a major factor in determining the fate of one penicillin as compared to another, the slope of these curves should have been different. Such was not the case.

3. Extending the time-plasma level curve back to zero permits a calculation of the apparent volume of distribution of each penicillin. For G this was estimated as 20.2% or corresponds roughly to total blood volume plus extra-cellular tissue fluid. In the case of penicillin K the apparent volume of distribution was 57.1% which at once suggested localization in tissues or some body fluid, other than blood.

Distribution of Penicillin G and K in Body

TABLE III.
Distribution of Penicillin G and K Between Dog Plasma and Saline After 20 Hr Dialysis at 4°C.

Concentration added to saline γ/ml	No. of exp.	Mean concentration after dialysis—γ/ml		Apparent % "bound"
		Plasma	Saline	
Na Penicillin G.				
3.0	8	3.6	2.3	46.7
30.0	6	46.3	28.6	36.1
Na Penicillin K.				
2.3	9	3.6	1.0	71.0
23.0	6	39.1	11.2	70.6

Fluids and Tissues After Continuous Infusion. Since calculation of the apparent volume of distribution of penicillin K suggested localization, it was obviously necessary to determine the distribution of the 2 penicillins in tissues. There are only a few data available in the literature on the distribution of penicillin in tissues of animals, and in all cases the studies[7,8] have been carried out with impure preparations. In our studies dogs were lightly anesthetized with sodium pentobarbital following which a cannula was inserted into a femoral vein and attached to a burette, so that an appropriate concentration of penicillin could be infused at a constant rate of 1 ml per minute. Plasma concentration was determined at the end of one hour and again at 2 hours, following which infusion was discontinued, the animal was killed and representative tissues removed and prepared for assay as described above. Each penicillin was administered in a dose which was calculated to maintain the plasma concentration between 15-20 μg per ml for the period of infusion so as to obtain equilibrium between plasma and tissues. To accomplish this, the rate of infusion of G was maintained at 0.1 mg per kg per minute. For K, 0.2 mg per kg per minute was required.

Results of these experiments are summarized by Table II. As was expected kidney had the highest concentration, whereas brain and spinal fluid had the lowest levels. Lung and liver had higher concentrations than were predicted on the basis of blood and tissue fluid content, thus suggesting some localization. The most striking difference in distribution of the 2 penicillins was noted in the liver assays. The concentration of K in this organ approached the concentration in plasma, and was nearly twice as high as that of G.

Adsorption of Penicillin G and K by Dog Plasma. The error in cup test assays of penicillin K in plasma described above indicated the likelihood of binding by some constituent of plasma. A cellophane bag containing 7.5 ml of plasma was suspended in a dialysis tube and equilibrated with 15 ml of saline containing a desired concentration of penicillin. Dialysis was carried out on a rocking machine at 4°C for 20 hours, following which penicillin concentration in each fluid was determined.

The results summarized in Table III show that in all instances the concentration of penicillin in plasma was higher than in the corresponding saline solution. Per cent of "bound" penicillin was calculated by the usual method of subtracting concentration in saline from concentration in plasma and dividing by total plasma concentration. Interpretation of the data is difficult because of an apparent high recovery of both penicillins in these experiments, which could not be explained by irregularities in the assay procedure. In spite of this there is little question but that K was bound to a far greater extent than G; thus furnishing a reasonable explanation for the difficulty of determining true concentration of penicillin K in plasma by the cup test procedure.

Discussion. The foregoing experiments leave little doubt as to the fact that penicillin K is handled by the intact animal in a quantitatively different manner than G. The fundamental basis for these differences is not clear at the moment in terms of the commonly recognized chemical properties of the 2 penicillins. However, data on the physical characteristic of these penicillins are not now available, and it may be that information along these lines will clarify the problem.

Regardless of the explanation of the differences in properties of these 2 penicillins, K has an apparent disadvantage for the treatment of systemic infections. This arises from the greater difficulty in maintaining a given total plasma concentration, and because it is likely that adsorption decreases the effective plasma level. Cup test assays may give a misleading picture of total penicillin K content in plasma. However, they may well give a truer indication of the concentration of "free" penicillin available for therapeutic effect.

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[7] Stuble, G. G., and Bellows, J. G., *J. A. M. A.*, 1944, **125**, 685.

[8] Cutting, W. C., Luduena, F. P., Fiese, M., Elliot, H. W., and Field, J., *J. Pharm. and Exp. Therap.*, 1945, **85**, 36.

Summary. 1. As determined by the customary cup test procedure it is not possible to obtain complete recoveries of penicillin G and K when added to plasma and tissues. A considerable error is involved in the assay of penicillin K in the presence of a high concentration of plasma. Such low recoveries have been shown not to be due to destruction of penicillin K. 2. For a given dose, the plasma level of penicillin K 5 minutes after intravenous injection is less than one-half that with penicillin G. The rate of disappearance from a given plasma concentration, however, is not greatly different for the 2 penicillins. 3. The apparent volume of distribution of G is 20.2% of the body weight, whereas with K it is 57.1%. Both of the penicillins are localized in lung and liver to some extent. Penicillin K is localized to a considerably greater extent in the liver than is G. 4. As determined by dialysis experiments penicillin K is adsorbed by some component of dog plasma to a much greater extent than is penicillin G.

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Subtilin-Antibiotic Produced by *Bacillus subtilis*.* V. Effect on *Streptococcus pyogenes* Infections in Mice.†

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In a previous communication[1] subtilin was found to exert a pronounced antibiotic action against a large number of species of Gram-positive organisms, including *Streptococcus pyogenes* (hemolytic) by the agar cup-plate procedure. The agent was shown to be bacteriostatic in high dilution and bactericidal in more concentrated solution. Subtilin exhibited an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro*[2] and exerted a powerful *in vivo* action on the course of experimental pneumococcus infections in mice[3] and anthrax in guinea pigs.[4]

TABLE I.
Treatment of *Streptococcus pyogenes* Infections in Mice.

		6 mice used in each group				
Date	Time	Control	Treated immediately	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
8-13-46	9 a.m.	Infected	Infected	Infected	Infected	Infected
,,	9 ,,	—	0.5 cc subtilin	—	—	—
,,	12 m.	—	,, ,, ,,	0.5 cc subtilin	—	—
,,	3 p.m.	—	,, ,, ,,	,, ,, ,,	0.5 cc subtilin	—
,,	6 ,,	—	,, ,, ,,	,, ,, ,,	,, ,, ,,	0.5 cc subtilin
,,	11 ,,	All dead	1.0 ,, ,,	1.0 ,, ,,	1.0 ,, ,,	1.0 ,, ,,
14	9 a.m.	—	,, ,, ,,	,, ,, ,,	,, ,, ,,	,, ,, ,,
,,	12 m.	—	,, ,, ,,	,, ,, ,,	,, ,, ,,	,, ,, ,,
,,	4 p.m.	—	,, ,, ,,	,, ,, ,,	,, ,, ,,	,, ,, ,,

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* This investigation was aided by a grant from Eli Lilly and Co., Indianapolis, Indiana.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

[1] Salle, A. J., and Jann, Gregory, J., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 60.

[2] Salle, A. J., and Jann, Gregory J., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 23.

[3] Salle, A. J., and Jann, Gregory J., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 40.

[4] Salle, A. J., and Jann, Gregory J., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 41.

TABLE II.
Treatment of *Streptococcus pyogenes* Infections in Mice.

		8 mice used in each group				
Date	Time	Control	Treated immediately	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
9-19-46	9 a.m.	Infected	Infected	Infected	Infected	Infected
"	9 "	—	0.5 cc subtilin	—	—	—
"	12 m.	—	" " "	0.5 cc subtilin	—	—
"	3 p.m.	—	" " "	" " "	0.5 cc subtilin	—
"	6 "	—	" " "	" " "	" " "	0.5 cc subtilin
"	8 "	1 dead	—	—	—	—
"	9 "	—	1.0 cc "	1.0 " "	1.0 " "	1.0 " "
9-20-46	9 a.m.	All dead	0.5 " "	0.5 " "	0.5 " "	0.5 " "

In the present communication subtilin was tested for its effect on *Streptococcus pyogenes* infections in white mice.

Experimental. Thirty white mice, weighing between 20 and 25 g were injected intraperitoneally with 0.2 cc of a saline suspension of a 24-hour brain heart infusion agar slant culture of *Streptococcus pyogenes*. The turbidity of the saline suspension was adjusted to correspond to a No. 3 MacFarland nephelometer standard.

The animals were divided into 5 groups and treated as follows: mice in Group 1 were not treated but served as controls; mice in Group 2 were treated immediately after infection; mice in Group 3 were treated 3 hours after infection; mice in Group 4 were treated 6 hours after infection; and the mice in the last group were treated 9 hours after infection. Each mouse was injected intraperitoneally with 0.5 cc of a solution containing 0.2 mg subtilin per cc (1 unit)[2] and every 3 hours thereafter. This dose was increased to 1 cc (2 units) at the end of the first day and throughout the next.

The schedule of treatments and results obtained are given in Table I. It may be seen that all of the control animals died within 14 hours whereas all of the treated animals survived and were apparently normal 2 weeks after treatment was discontinued. The mice were discarded after this period of time.

The animals treated 9 hours after being infected were 5 hours from death and appeared to be beyond recovery. The infection was accompanied by diarrhea, ruffled fur, and prostration. After receiving 2 injections of subtilin, the diarrheal condition disappeared and the animals appeared to be almost normal. After the fourth injection, the animals looked and acted like healthy mice and devoured food freely. The recovery was so spectacular that it was almost beyond belief.

In a second series a larger number of mice (40) was used to check the results in the first experiment. The method was the same but the amount of subtilin administered was less. The results are recorded in Table II. It may be seen that one of the untreated mice died within 11 hours. The others died within 24 hours but the exact time could not be ascertained because death occurred sometime during the first night. On the other hand, all of the animals given subtilin survived and were healthy and normal 2 weeks after treatment was discontinued. The animals were sacrificed after this period of time. Examination of the hearts' blood of animals dead of the disease showed the presence of large numbers of *Streptococcus pyogenes*. The antibiotic did not produce any observable toxic reaction in the mice.

Conclusions. Subtilin has been shown to exert a powerful *in vivo* action on the course of experimental *Streptococcus pyogenes* infections in mice. Animals given subtilin 9 hours after being infected were in such an advanced stage of the disease at the time treatment was started that recovery was almost unbelievable. The antibiotic exhibited no apparent toxic reaction in the mice.

15657 P

Biotin Deficiency in the Dairy Calf.[*]

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During the course of the experiments on the development of a synthetic ration for the dairy calf (described elsewhere),[1] 2 of the animals developed a paralysis that was cured by the administration of biotin. This syndrome occurred when the animals were fed Ration No. 2. The composition of the diet is given in Table I.

The lard was homogenized into the solution of casein, salts and cerelose to produce a "synthetic milk" which contained 13% solids. All vitamin supplements were given at the morning feeding. For complete details on the preparation of the diet see Wiese, Johnson, Mitchell and Nevens.[1]

The first animal, calf No. 7, having been on Ration 2 for 19 days, developed a paralysis of the rear legs and was unable to rise. The calf, very unsteady when placed on his feet, was incapable of muscular coordination of the hind legs and fell when trying to walk.

An attempt was made to cure the condition by giving the animal 25 g of Wilson's 1-20 liver powder in the morning feeding. A slight improvement was shown by afternoon. The following morning, the paralysis had disappeared, and the calf was able to rise without assistance and walk normally. The animal, continued on Ration 2, exhibited the same paralytic symptoms 22 days later. The calf was given 100 μg of biotin by subcutaneous injection. In 24 hours the symptoms had disappeared and the animal had recovered completely.

Calf No. 7 developed the identical symptoms a third time 13 days later and was cured again by the subcutaneous injection of 100 μg of biotin.

A second animal, calf No. 9, having been on the diet for 50 days, showed the same symptoms as calf No. 7. The calf was given 100 μg of biotin by subcutaneous injection. When the condition of the animal did not improve, 1 mg of biotin in glucose was administered by intravenous injection. The next day the animal had recovered completely.

TABLE I.
Composition of Ration 2.

Component	%	Vitamin added	Mg/kg liquid ration
Casein "Labco"	30.0	Thiamin	0.65
Lard	26.6	Riboflavin	0.65
Salts 1	6.0	Nicotinic acid	2.60
Cerelose	37.4	Pyridoxine	0.65
		Calcium pantothenate	1.30
		Ascorbic acid	13.0
		α-Tocopherol	1.0
		2-Methyl-1,4-naphthoquinone	0.26
		Vitamin A	5000 I.U./day
		Vitamin D	500 I.U./day

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[†] Present address: Department of Agricultural Chemistry, University of Idaho.

[1] Wiese, A. C., Johnson, B. Connor, Mitchell, H. H., and Nevens, W. B., *J. Dairy Sci.*, in press.

TABLE II.
Urinary Excretion of Biotin.

Date	Symptoms	Treatment	Excretion γ/day	Date	Symptoms	Treatment	Excretion γ/day
		Calf 7.				Calf 9.	
Born 1-28-46				Born 2-12-46			
2-12			5.2	2-23	—		1.9
13	—		4.4	24	—		3.1
17	+			4-5	+	100 γ biotin	—*
18	+	25 g liver	—*	6	—		
19	—						
3-12	+	100 γ biotin	—*	18	—	On starch containing diet	10.5
13	—						
25	+	100 γ biotin	—*	19	—		15.1
26	—			5-17	—		25.6
4-1	—	On starch containing diet		29	—		10.7
				6-6	—		21.2
5-4	—		14.6				
29	—		10.6				
		Calf 11.				Calf 12.	
Born 4 24-46				Born 4-24-46			
4-26	—	On colostrum	46.4	4-26	—	On colostrum	35.1
5-3	—	On synthetic milk diet	4.4	5-3	—	On synthetic milk diet	2.1
4	—		5.3	4	—		3.9
9	—		2.5	9	—		3.2
10	—	+ biotin†	5.7	10	—	+ biotin†	8.2
18	—		7.3	18	—		5.4
24	—		7.6	24	—		4.8
29	—		10.6	6-1	—		12.6
6-4	—		14.3				

* Urine samples not obtained due to scouring.
† Biotin added to the synthetic milk'' diet at the rate of 10 μg per 1000 cc of milk.

Calves No. 7 and 9 were changed to a solid diet containing considerable amounts of starch when 7 weeks old. On this diet the animals did not exhibit any paralytic symptoms.

During the course of the experiment 24-hour urine collections were made. The amount of biotin excreted was determined by the method of Wright and Skeggs.[2] The data are given in Table II. At the age of 2 weeks, calves No. 7 and 9 excreted only a small amount of biotin in the urine. The increase in biotin excretion by these animals during the latter stages of the experiment is probably due to the change to a solid diet containing considerable amounts of starch. On this type of ration intestinal synthesis of the B-vitamins, including biotin, probably occurred, accounting for the increased biotin excretion in the urine.

Calves No. 11 and 12 were maintained on the liquid "synthetic milk" diet. Biotin was not supplemented for the first 2 weeks. After this time 10 μg of biotin were added per kg of liquid ration fed. The first urine sample was obtained when the calves were 48 hours old and had received colostrum. The data in Table II reveal that the biotin excretion was high at this time. After one week on the biotin-deficient ration the urinary biotin excretion had dropped to 3 to 5 μg per day. After the supplementation of biotin was begun, there was a gradual increase in the urinary excretion of biotin. These 2 calves grew normally and did not show any symptoms of biotin deficiency during the time they were on the experiment.

Summary. The young dairy calf requires an exogenous supply of biotin; otherwise a paralysis of the hind quarters develops, curable by biotin administration.

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[2] Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**. 95.

15658

Changes Produced by Starvation in the Vitamin Content of Rat Tissues.

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(Introduced by Ralph R. Mellon.)

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It has been observed in this laboratory that the topical application of β,β'dichlorоethyl sulfide (mustard gas) in rats causes marked changes in the vitamin content of some tissues. Since severe starvation is a prominent feature of mustard gas poisoning, it was deemed necessary to determine the effect of starvation *per se*. This report describes the effects of mustard gas and severe starvation upon the liver, kidney, and bone marrow contents of 6 of the B vitamins.

Methods. Animals. Twenty-five-day-old, male, albino rats of the Sprague-Dawley strain were housed in individual wire-mesh cages and were fed the following ration: casein (Labco, "vitamin-free") 18; sucrose, 76; salts IV,[1] 4; and corn oil, 2. To each kilogram of ration were added: thiamine hydrochloride, 3.0 mg; riboflavin, 3.0 mg; pyridoxine hydrochloride, 3.0 mg; calcium pantothenate, 10.0 mg; *i*-inositol, 0.3 g; choline chloride, 1.0 g; and 2-methyl-1,4-naphthoquinone, 10.0 mg. At the beginning of the experiment, the rats were given one drop of halibut liver oil (Abbott-plain) containing 1.3 mg of added *dl*-α-tocopherol acetate. All of the rats received this ration *ad libitum* for 4 days, at which time the experimental procedures were initiated.

Tissues. At the conclusion of the experiments the rats were decapitated, and the tissues removed for analysis. The total weights of the livers were determined, and weighed portions of both the liver and kidney were dried for 48 hours at 45°C *in vacuo*. The samples were reweighed to determine the dry weights, then stored in the cold until prepared for analysis. The dried livers and kidneys were ground and a weighed portion (approximately 50 mg) homogenized in 10 ml of water with a glass homogenizer.[2] Each sample was incubated at 37°C under toluene with clarase and papain (each enzyme added at a level equivalent to 5% of the weight of the tissue) for 24 hours at the natural pH, and then for 24 hours in .05 N sodium acetate buffer at pH 4.5.

The 6 long bones (femora, tibiae, and humeri) were split with a scalpel and the marrow, which was removed with a hypodermic needle filed on one side to form a scoop, was suspended in water and stored in the cold under toluene until prepared for assay. The bone marrow was homogenized and the nitrogen content determined on an aliquot by a micro digestion and nesslerization procedure.[3] One gram of each of the enzymes per gram of nitrogen was added and the tissue digested as above, except that .02 N sodium acetate buffer was used.

In all the tissues, the digests were adjusted to pH 6-7, steamed and rehomogenized. These preparations were used for the assays of vitamin B_C and pantothenic acid; for biotin determinations an aliquot was autoclaved at 120°C for 2 hours in 6 N sulfuric acid and neutralized; for nicotinic acid an aliquot was autoclaved at 120°C for 20 minutes in 1 N sulfuric acid and neutralized; and for riboflavin and pyridoxine an aliquot was steamed at 100°C for one-half hour in 0.1 N hydrochloric acid, adjusted to pH 4.5 with sodium acetate, and filtered.

Vitamin Assays. The analyses of these tissues for their content of vitamin B_C (employing *L. casei* with the vitamin B_C reference standard of Parke, Davis & Co.), biotin, pantothenic acid, nicotinic acid, and riboflavin were performed by modifications of the

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[1] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[2] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[3] Johnson, M. J., *J. Biol. Chem.*, 1941, **137**, 575.

TABLE I.
Constituents of Media for Microbiological Assays.

Constituent	Amt./liter double-strength medium	
	Lactobacilli	*S. carlsbergensis*
Casein hydrolysate* (SMACO, 10% soln.)	100.00 ml	80.00 ml
Alkali treated peptone†	10.00 g	—
Yeast extract supplement†	2.00 "	—
Norit treated peptone‡	0.40 "	—
dl-Alanine‡	0.40 "	—
dl-Tryptophane	0.40 "	—
l-Asparagine	0.20 "	—
l-Cystine	0.20 "	—
Glucose	40.00 "	40.00 g
Sodium acetate (anhyd.)	24.00 "	—
Potassium citrate · H_2O§	1.00 "	10.00 "
Citric acid · H_2O	—	2.00 "
K_2HPO_4	1.00 "	0.60 "
KH_2PO_4	1.00 "	0.60 "
$MgSO_4 \cdot 7H_2O$	0.40 "	0.20 "
$FeSO_4 \cdot 7H_2O$	20.00 mg	10.00 mg
$MnSO_4 \cdot 4H_2O$	20.00 "	10.00 "
NaCl	20.00 "	10.00 "
Adenine sulfate	20.00 "	—
Guanine hydrochloride	20.00 "	—
Uracil	20.00 "	—
Xanthine	20.00 "	—
i-inositol	—	20.00 "
Nicotinic acid	2.00 "	—
Pyridoxine hydrochloride	2.00 "	—
Calcium pantothenate	1.00 "	2.00 "
Riboflavin	1.00 "	—
Thiamine hydrochloride	1.00 "	1.00 "
p-Aminobenzoic acid	0.10 "	—
Biotin	0.01 "	0.01 "
Adjust to pH	6.5-6.6	5.2-5.5

* Omitted in the riboflavin assay.[8]
† Added only in the riboflavin assay.[8]
‡ Added only in the vitamin B_c assay.[4]
§ Prevents precipitation of the mineral salts in the *Lactobacilli* assays.

existing microbiological methods.[4-8] The various media proposed for assays with the *Lactobacilli* have been incorporated into a single medium which, with the modifications indicated, serves for all of the *Lactobacilli* assays. The constituents of the medium are given in Table I, requiring only the omission of the vitamin under consideration for its assay. The assays employing the *Lactobacilli* were carried out in 5 ml quantities and inoculated by the convenient procedure suggested by Black and Arnold.[9] The *L. casei* were incubated at 37°C and the *L. arabinosus* at 30°C for 3 days and titrated electrometrically with 0.05 N NaOH. Pyridoxine was determined by the *S. carlsbergensis* method[10] employing the medium shown in Table I. The assays were run in 5 ml quantities contained in 50 ml Erlenmeyer flasks. The flasks were incubated for 18 hours at

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[4] Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, **157**, 303.

[5] Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 95.

[6] Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

[7] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

[8] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

[9] Black, T. L., and Arnold, A., *Ind. and Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

[10] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

30°C and growth was determined turbidimetrically in the Evelyn colorimeter.

Vitamin B_C assays on bone marrow samples extracted with ether at pH 4.5[11] indicated that fatty materials did not interfere with the assay. Recovery experiments on bone marrow for vitamin B_C and on liver for vitamin B_C and pantothenic acid also showed that the assays were functioning satisfactorily. The vitamin B_c contents of some samples of liver and bone marrow were also determined by the *Streptococcus fecalis R* method.[12] These values were 10-20% lower than those obtained by the *L. casei* method. Assays on the enzymes used for digestion of the tissues showed that there were insufficient quantities of the vitamins present to necessitate any correction in the values obtained.

The vitamin content of the livers and kidneys was expressed in amount per gram of dry tissue; while that of the bone marrow was expressed on the basis of amount per gram of nitrogen. We chose to express our vitamin concentrations per gram of nitrogen in the case of bone marrow since the constituents of this tissue are frequently expressed on the basis of nitrogen or phosphorus and because this procedure is experimentally convenient for bone marrow.

Experimental. Each of the experiments presented in this paper (Series I and Series II) consisted of 3 groups of animals: a *mustard-treated* group (fed *ad libitum*) which received topically 8 mg of mustard gas per kg of body weight and 2 *control* groups, one of which was fed *ad libitum* while the food allowance of the other was restricted to varying degrees as described below. Mustard gas was not administered to the control groups. The daily food consumption of each animal was recorded. The average weight of the animals at the beginning of the experimental period was 50 g in Series I and 46 g in Series II.

Series I. In the *mustard-treated* group, analyses were performed upon the tissues of 8 animals which died 3-7 days after the application of mustard. The average daily food consumption of these animals was 3.4 g and the average daily loss in weight was 3.1 g.

The food intake of each rat in the *control-restricted* group (8 rats) was limited to that consumed during the previous 24 hours by its paired member in the mustard group. Each animal was sacrificed 24 hours after the death of its paired-fed mate. The average daily loss of weight in this group was 1.3 g with considerable variation among animals in the total loss of weight over the 3-7-day period. The average daily food consumption of the *control-ad libitum* group (8 rats) was 8.5 g and the average daily weight gain was 3.2 g. The animals were sacrificed on the fifth and sixth days of the experiment.

In general, the concentrations of vitamins in tissues from the mustard-treated and food-restricted groups were similar and differed from those of the groups fed *ad libitum*. These changes were particularly evident in the liver, where marked increases were noted. The changes observed in this series were similar to those tabulated for Series II (Table III). Of significance for the purposes of this report is the fact that the deviations from the normal in the partially-starved group could be correlated with the degree of weight loss of the animals. This was particularly true for the concentrations of vitamin B_C which increased in the liver and decreased in bone marrow. As mentioned previously, the weight changes in the members of this group were variable and could be accounted for by the variations in the food intakes of their partners in the mustardized group. It was also apparent that the utilization of food by the rats poisoned with mustard was impaired to varying degrees in individual members of this group. Thus, the weight losses in these animals were uniform, in spite of the differences in food intake. These observations led to the conclusion that the vitamin changes in the mustard-treated animals were secondary to inanition and that the extent of the changes in the paired-fed rats was conditioned by the weight loss of the animals. Accordingly, in Series II, the food allowance of one control group was

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[11] Bauernfeind, J. C., Sotier, A. L., and Boruff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 666.

[12] Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

TABLE II.
Changes in Liver Size Resulting from Mustard Treatment or Severe Starvation for 4 Days (Series II).*

Group	Starting wt (g)	Final wt (g)	Wt of liver (g)	% liver†
Control—*ad lib.*	46	54	3.10	5.74
(10 rats)	(40-51)	(45-59)	(2.35-4.58)	(5.06-6.18)
Control—restricted	46	35	1.22	3.48
(10 rats)	(39-50)	(27-39)	(0.69-1.67)	(2.56-4.00)
Mustard-treated	44	34	1.30	3.82
(10 rats)	(40-46)	(31-36)	(1.03-1.60)	(2.86-4.56)

* Averages are given with the range of values in parentheses.
† Calculated from the final body weight.

TABLE III.
Effects of Mustard Treatment and of Severe Starvation upon the Concentrations of Vitamins of Rat Tissues (Series II).*

Group	Vitamin B_c	Pantothenic Acid	Biotin	Riboflavin	Pyridoxine	Nicotinic Acid
			Liver.			
Control—*ad lib.*	7.02	225	2.31	88.3	16.2	377
	(5.50-8.30)	(151-304)	(1.50-3.82)	(62.4-116)	(10.4-24.8)	(334-426)
,, —Restricted	19.7	428	5.01	134.	42.6	554
	(10.9-30.3)	(378-515)	(3.70-6.62)	(110-164)	(35.8-53.4)	(513-640)
Mustard-treated	24.3	433	4.71	131.	39.5	546
	(14.7-33.6)	(326-540)	(3.70-5.98)	(106-147)	(34.2-46.5)	(505-589)
			Kidney.			
Control—*ad lib.*	11.1	158	3.17	112.	15.7	300
	(7.78-13.6)	(146-168)	(2.28-3.76)	(104-124)	(11.4-22.0)	(280-320)
,, —Restricted	14.4	260	4.94	111.	25.4	310
	(7.20-23.9)	(218-317)	(3.98-5.90)	(87.0-146)	(18.7-32.0)	(283-338)
Mustard-treated	16.2	293	5.04	104.	23.3	306
	(9.30-27.8)	(243-356)	(4.36-6.62)	(91.0-117)	(18.8-27.9)	(290-335)
			Bone Marrow.			
Control—*ad lib.*	26.1	360	1.56	262.	36.2	1033
	(22.4-30.4)	(322-451)	(1.05-2.18)	(216-296)	(16.7-67.6)	(815-1325)
,, —Restricted	16.7	332	1.31	359.	63.2	1079
	(14.3-20.2)	(262-430)	(0.73-1.86)	(200-547)	(35.6-93.0)	(953-1250)
Mustard-treated	8.8	381	1.32	263.	42.5	1220
	(4.9-14.6)	(285-500)	(0.54-1.82)	(171-398)	(32.4-50.7)	(940-1570)

* The liver and kidney values are expressed as micrograms of vitamin per gram of dry weight; the bone marrow values are expressed as micrograms of vitamin per gram of nitrogen. Values given represent the averages of determinations on 10 samples, with the exception of vitamin B_c in bone marrow, in which case 20 samples were analyzed. Ranges are given in parentheses.

limited to amounts which kept the weight losses on a parity with those of the mustardized rats.

Series II. In the mustard-treated group, analyses were made of the tissues of animals which died on the fifth day after mustard gas application. The animals in the *restricted* group were each given 2 g of control ration daily for 3 days and no food on the fourth day. The following day the animals in this group, as well as those in the control group fed *ad libitum* were sacrificed. This degree of food restriction resulted in weight losses equal to those of the mustardized rats (Table II). This experiment was repeated exactly in order to obtain sufficient bone marrow to complete the vitamin analyses. The vitamin contents of the liver, kidney, and bone marrow from 10 animals of each group were determined, with the exception of the bone marrow vitamin B_C in which case 20 tissues from each group were analyzed.

Weight changes in the 3 groups, along with the total liver weight and the percentage of liver in the rats (based on final body weight) are given in Table II for purposes of calculations to be discussed. The vitamin contents of the tissues are given in Table III.

Neither mustard treatment nor starvation produced any change in the percentage moisture content of the livers or kidneys.

Discussion. The foregoing data (Table III) demonstrate that severe starvation produces a marked rise in the content per gram of tissue of several of the B vitamins in the liver and that these changes also occur in the kidney, though to a lesser extent. The only notable change in the bone marrow is the fall in the vitamin B_C content. Although the riboflavin and pyridoxine values in the bone marrow show a trend in the opposite direction, it must be pointed out that the values in the starved and normal groups overlap to a great extent. Increases in the succinoxidase[13] and nucleoprotein[14] contents of liver during starvation have been reported.

It is also apparent (Table III) that the concentrations of the vitamins in the starved group are very similar to those in the mustard-treated group. It appears, therefore, that the changes in the vitamin content induced by mustard treatment are secondary to the concomitant inanition. The effect of mustard upon the vitamin B_C content of the bone marrow, however, appears to be specific. In this case the decrease in the vitamin due to the mustard treatment cannot be accounted for solely by inanition. This observation is of interest in view of the well-known deleterious effect of mustard upon the leukopoietic activity of the bone marrow. The prominent role of vitamin B_C in leukopoiesis suggests that the action of mustard upon the bone marrow is exerted through its effect upon the metabolism of vitamin B_C.

Table II shows that both mustard treatment and starvation cause a considerable decrease in liver size, both on an absolute basis and expressed as percentage of body weight. Under such circumstances it becomes necessary to reassess the significance of values based upon a unit weight of dry tissue. Two alternate methods of calculation present themselves: (a) determination of the total content of the constituent in the liver; or (b) calculation of the liver content per 100 g of body weight (percentage basis). Various investigators have elected to calculate the amounts of the following constituents in terms of one of these methods: vitamin,[15] enzyme,[13,16,17] nucleoprotein and phospholipid.[18]

Comparisons based upon the total amount of the constituent in the liver would appear to be valid only when the weights of the various groups of animals are the same. On the other hand, the expression of values in terms of the liver constituent per 100 g of body weight compensates for slight differences in body weight. Since the ratio of liver weight to body weight in normal rats varies during growth, such a calculation when applied to growing rats should be employed only when the ages of the different groups of animals under comparison are similar.

We believe that, under the conditions of the present experiment, calculations based upon the amount of liver constituent per 100 g of body weight furnish the most reliable basis for evaluating the significance of our data. Such calculations made from data for the liver given in Tables II and III (using amount of vitamin per unit of dry weight since the percentage dry weight is the same in the 3 groups) demonstrate that both starvation and mustard treatment cause increases ranging from 15 to 132% in the vitamin B_C, pantothenic acid, biotin and pyridoxine contents. No changes in the riboflavin and nicotinic acid values are observed. It is of interest that in the kidney, where the vitamin values are expressed per unit of dry weight of tissue, riboflavin and nicotinic acid are also the only 2 vitamins whose contents are not affected by starvation or mustard.

It is evident that the effects of inanition upon the contents of various liver constituents must be considered in cases where severe starvation for a short period of time results from a given set of experimental conditions. This factor is of particular significance when one is dealing with pharmacological agents which often produce such a state of severe starvation.

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Summary. 1. The vitamin B_C, pantothenic acid, biotin, riboflavin, pyridoxine, and nicotinic acid contents of the livers, kidneys, and bone marrows have been determined in rats: (a) fed *ad libitum;* (b) treated with mustard gas; and (c) whose food intake was restricted. 2. When expressed on the basis of unit weight of tissue, increases of 50 to 180% were noted in the vitamin concentrations of livers from the food-restricted group. Less marked increases occurred in the kidney, while a decrease of 35% in the vitamin B_C content of the bone marrow based on nitrogen content was observed. 3. With the exception of the vitamin B_C content of the bone marrow, the changes observed in the partially-starved group were also found in the mustard group. 4. Calculations based upon the vitamin content of the liver per 100 g of body weight indicate that both starvation and treatment with mustard gas cause increases ranging from 15 to 130% in the vitamin B_C, pantothenic acid, biotin, and pyridoxine contents of the liver. No significant changes occur in the riboflavin or nicotinic acid contents.

We are indebted to the Parke, Davis and Company for the vitamin B_c reference standard; and to Merck and Company for the synthetic vitamins. This work was done under a contract from the Medical Division of the Chemical Warfare Service.

15659

Toxicity of Amino Acids as Influenced by Riboflavin Deficiency.

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The therapeutic application of protein hydrolysates has become common and as a result it grows more and more important to understand interrelationships between amino acids, vitamins, minerals, etc. In the course of experiments involving toxicity studies of histidine[1] and tyrosine,[2] it became probable that riboflavin deficiency was a key to increased amino acid toxicities. To establish this point, histidine and tyrosine toxicities were rechecked and cystine, glycine, glutamic acid and tryptophane were investigated.

Experimental. The various amino acids were incorporated into a synthetic diet[3] at varying levels from 5 to 20% depending upon the relative toxicity of the amino acid under consideration. The amino acid replaced an equivalent amount of carbohydrate in the basic diet. Glycine, *l*-tyrosine, *l*-histidine, *l*-cystine, *d*-glutamic acid and *dl*-tryptophan were used. The rats in all series showed crusted and bloody snouts, spectacled eyes, and diarrhea. The animals receiving glycine with or without superimposed riboflavin deficiency lost their hair; some became almost hairless. The rats were lethargic and appeared to lack muscular coordination. In those receiving glycine without a superimposed B_2 deficiency the hair condition corrected itself after a few weeks. Emaciation was common.

The symptomatology seen in these rats on toxic levels of an amino acid is also seen in riboflavin deficiency[4] but it should be recalled that these manifestations are nonspecific in nature.

Table I summarizes our results.

Accentuation of the toxicities of amino

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TABLE I.
Toxicity of Amino Acids as Influenced by Riboflavin Deficiency.
10 rats to each set.

	% dead Months			Avg wt, g Months		
Diet	0	1	2	0	1	2
5% Tyrosine	0	0	0	47	84	162
” ” Riboflavin def.	0	70	100	46	60	70
10% Histidine	0	30	40	46	68	82
” ” Riboflavin def.	0	80	100	42	63	—
5% Cystine	0	0	20	51	102	173
” ” Riboflavin def.	0	60	80	48	78	64
20% Glycine	0	20	40	50	74	98
” ” Riboflavin def.	0	60	100	53	46	—
10% Glutamic acid	0	20	40	48	115	198
” ” ” Riboflavin def.	0	20	20	47	78	84
20% *dl*-tryptophane	0	20	40	42	75	63
” ” Riboflavin def.	0	20	80	49	41	32
Riboflavin def.	0	0	10	41	78	89

acids occurred without exception in the 6 instances reported. This is established both by the increased death rate and by the decreased growth responses seen in the riboflavin-deficient animals. Tyrosine (5%), histidine (10%), and glycine (20%) were the most toxic amino acids in the riboflavin-deficient animals; whereas histidine (10%), glycine (20%), glutamic acid (10%) and tryptophan (20%) were the most toxic in the rats receiving riboflavin.

Discussion. The toxicity of tyrosine,[3,5] histidine,[2,6] glycine,[7] tryptophan,[5] cystine,[7] lysine,[5] serine,[8] methionine,[9] leucine,[6] hydroxyproline,[6] aspartic acid[6] has been reported. Glutamic acid is now added to this list. The results in this paper indicate increased toxicities for glycine, tryptophan, glutamic acid, cystine, histidine and tyrosine in the presence of a riboflavin deficiency. Balance is the deciding factor but there is specificity in these balance relationships as shown by the increased toxicity of tyrosine in a riboflavin deficiency[1] with no similar result in thiamin, pantothenic acid, folic acid, pyridoxine and vitamin A and D deficiencies. It is reflected in the ability of certain amino acids to counteract increased toxicities while others do not. As an example of this cystine decreased the toxicity of tyrosine,[1] while tryptophan and glutamic acid have no effect.

The explanation offered for consideration in the specific action of riboflavin deficiency in increased amino acid toxicities is that *l*-amino acid oxidase probably is a riboflavin-containing enzyme.[10]

Summary. *l*-Cystine, *dl*-tryptophan, *l*-tyrosine, *l*-histidine, glycine, and *d*-glutamic acid are more toxic in the riboflavin-deficient rat than in the rat receiving this vitamin.

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15660

Gonadotrophic Potency of Ewe Pituitary Glands as Affected by Spaying, Season, and Breed.*

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The breeding season in ewes of most breeds is limited to the fall and early winter months.[1-4] During the remainder of the year, the ewe is anestrual although the ovaries often show a certain amount of activity, especially during the last few weeks before the breeding season begins.[1]

A lowered activity on the part of the pituitary gland has been postulated as a possible reason for anestrus in the ewe since injections of gonadotrophic hormones from pregnant mare serum or pituitary glands produce ovulation in a high percentage of anestrual ewes.[1,5] The great variability in response to injections of this kind,[6,7] however, raises a question as to whether other physiological factors may be involved.

The gonadotrophic hormone content of the pituitary gland of the male 13-lined ground squirrel increases greatly during the breeding season,[8] suggesting that at least in this species hormone content of the gland is correlated with secretory rate and the activity of the gonads. The gonadotrophic hormone content of the pituitary gland of the male cottontail rabbit increases during the breeding season, but that of the female does not.[9] The gonadotrophic hormone content of ewe pituitary glands remains about the same during the breeding season and during anestrus,[10] suggesting that a lack of pituitary gland activity may not be wholly responsible for anestrus in the ewe.

The time of the breeding season is evidently dependent upon environmental factors since it shifts when ewes are taken to the southern hemisphere.[5] The success which has been achieved in changing the breeding season by modifying the amount of light received is further evidence in this regard.[11] The physiological mechanism through which environmental factors operate to control sexual activity in the ewe has never been studied.

The hormonic secretions of active gonads normally lower the gonadotrophic hormone content of the pituitary gland and a pronounced rise occurs after gonadectomy.[12] Working upon the postulate that the pituitary gland of the anestrual ewe would indicate its potential activity by its ability to produce and store gonadotrophic hormones after spaying, the experiment reported here was performed in an effort to elucidate some of the factors responsible for the anestrual condition.

Materials and Methods. The experimental animals were 7-year-old ewes, half of each

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group being purebred Hampshires and half Lincoln X Rambouillet crossbreds. A group of 8 ewes was spayed in September, 1944, and maintained for a period of 90 days with a group of similar ewes serving as controls. At the end of this period, both groups were slaughtered and the pituitary glands removed for assay. All ewes in the spayed group had active corpora lutea in their ovaries at the time of the operation, and the control ewes had corpora lutea indicating recent ovulations at the time of slaughter. Thus, all ewes appeared to be in a reproductive condition normal for the breeding season.

A group of ewes from the same band was bred in the fall of 1944 and showed that they were reproductively normal by producing lambs during February and March of 1945. The lambs were weaned April 1, 1945 and on April 13 a group of 8 ewes was spayed. They too were maintained with a group of similar ewes which served as controls. All ewes were slaughtered after a period of 90 days and the pituitary glands removed for assay. At the time of spaying, all the ovaries removed were inactive with no follicle larger than 5 mm in diameter being observed. During the period from spaying until slaughter, an active painted teaser ram was kept with the control ewes and none showed an estrual period. Thus, all ewes were anestrual in a manner typical of the season. There was some ovarian activity since, at autopsy, one of the control ewes was found to have a fresh corpus luteum and 4 others had follicles as large as 8 mm in diameter. Three of 15 other ewes from the same band, slaughtered the same day, were found to have corpora lutea.

The pituitary glands were removed within 30 minutes after slaughter and immediately placed in an excess of acetone.† After being taken to the laboratory, usually a matter of from 4 to 5 hours, as much of the adhering connective tissue as possible was removed, the glands cut into small cubes, and the acetone changed. Three additional changes of acetone were made at intervals of 24 hours, after which drying was completed in desiccators containing calcium chloride.

After drying, the individual glands were ground with a mortar and pestle until fine enough to pass through an 80-mesh (to the inch) screen.

Assays of individual pituitary glands were carried out with rats and chicks. In the rat assays, the increase in ovarian weight of 21-day-old females was used as an endpoint. A total dosage of 20 mg of pituitary powder plus 4 mg of recrystallized hemin as an augmenter was suspended in 4½ cc of distilled water containing enough 1% NaOH to dissolve the hemin and injected subcutaneously in 9 equal injections over a period of 4½ days. Autopsies were performed 14 to 16 hours after the last injection. In the chick assays, the increase in testis weight of day-old White Leghorn cockerels was used as an endpoint. A total dosage of 10 mg of pituitary powder was suspended in 2½ cc of distilled water and injected subcutaneously in equal injections once per day over a 5-day period. Autopsies were performed 24 hours after the last injection.

The plan was to assay each gland on 2 rats and 5 chicks, but due to some death losses among the assay animals during the injection period, a few mistakes in sexing of the chicks, and a shortage of powder in 2 cases, data were actually available on a smaller number in some cases.

Results and Discussion. The results show that with both rats (Table I) and chicks (Table II) as assay animals the pituitary glands of spayed ewes of both breeds produced much greater responses than did the glands of the intact control ewes of the same breed. As indicated in Table III, these differences were highly significant. The differences between seasons were not significant in either breed in either spayed or control groups. There was no significant overall difference between seasons and the season-spaying interaction did not approach significance. Thus, the increase in gonadotrophic potency of the ewe pituitary glands after spaying was apparently of about the

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† The cooperation of Armour and Co., Spokane, Washington, in making possible the collection of the glands in their plant is gratefully acknowledged.

TABLE I.
Ovarian Responses of Immature Rats to Injections of 20 mg of Pituitary Powder and 4 mg of Recrystallized Hemin.

Season in which donor ewes slaughtered	Type of donor ewes					
	Intact			Spayed		
	No. ewes	No. rats	Avg ovarian wt. mg	No. ewes	No. rats	Avg ovarian wt. mg
Breeding season:						
Hampshire	4	8	15.0 ± 1.3*	4	7	34.1 ± 17.3
Crossbred	4	7	27.9 ± 8.2	4	8	134.5 ± 45.0
Anestrus:						
Hampshire	4	8	13.4 ± 1.8	4	8	36.0 ± 8.2
Crossbred	4	8	20.0 ± 5.7	4	8	83.7 ± 19.8

* Standard errors based on between ewe mean squares.

TABLE II.
Testicular Responses of Immature Chicks to Injections of 10 mg of Pituitary Powder.

Season in which donor ewes slaughtered	Type of donor ewes					
	Intact			Spayed		
	No. ewes	No. chicks	Avg testis wt. mg	No. ewes	No. chicks	Avg testis wt. mg
Breeding season:						
Hampshire	4	17	17.4 ± 2.4*	4	17	19.5 ± 2.8
Crossbred	4	16	19.1 ± 3.0	4	17	25.2 ± 4.6
Anestrus:						
Hampshire	4	12	14.0 ± 2.9	4	13	22.1 ± 2.8
Crossbred	4	13	18.0 ± 2.7	4	14	22.1 ± 1.6

* Standard errors based on between ewe mean squares.

TABLE III.
Significance of Various Factors Influencing the Gonadotrophic Potency of Ewe Pituitary Glands as Determined by Analysis of Variance.

	Type of assay	
	21-day-old female rat	Day-old cockerel
Season	N.S.	N.S.
Breed	**	*
Spaying	**	**
Season × Breed	N.S.	N.S.
Season × Spaying	N.S.	N.S.
Breed × Spaying	N.S.	N.S.
Season × Breed × Spaying	N.S.	N.S.

Meanings of symbols are as follows:
N.S.—Not statistically significant.
*—Statistically significant P <.05.
**—Statistically highly significant P<.01.

same order during anestrus as during the breeding season.

The pituitary glands of the Lincoln × Rambouillet crossbred ewes were consistently more potent than those of Hampshire ewes, and as indicated in Table III, the differences were significant or highly significant as judged by the chick or rat assays, respectively.

The variances associated with the group means in the chick assay data were essentially homogeneous within the various groups and therefore well suited to the use of analysis of variance without transformation. The variances in the rat assay data were distinctly heterogeneous, and it was necessary to transform the ovarian weights to the logarithms of their logarithms before the mean squares seemed independent.‡

Qualitative as well as quantitative differences may exist between glands in their gonadotrophic hormone content. The degree of luteinization in the ovaries of the test rats was observed to be related to ovarian weight. Whether this represented a qualitative difference or merely a dosage effect is not known. Glands from spayed ewes in the 2 seasons were very comparable in their ability to produce luteinization.

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‡ The interest and cooperation of Professor George W. Snedecor, Iowa State College, Ames, Iowa, in suggesting and making these transformations is gratefully acknowledged.

These data show that the pituitary gland of the anestrual ewe is comparable to that of the breeding-season ewe in having the ability to store gonadotrophic hormones in increased amounts following spaying. Thus the anestrual ewe has the ability to produce appreciable amounts of these hormones, and the suggestion is strong that this ability may be as great as in the breeding-season ewe. This latter point cannot be considered as proven, however, since the amount of hormone stored in the gland may reach an equilibrium in less than 90 days. The data provide no information on the rate of increase.

In view of the apparent ability of the anestrual ewe to produce gonadotrophic hormones, the current theory that anestrus is due to a lowered activity on the part of the pituitary gland may have to be modified. Quite possibly the immediate cause of the anestrual season is some changed physiological condition outside the pituitary gland which influences the ability of the ovary to respond to gonadotrophic hormones. Any attempt to discuss possible factors at this time would be mere speculation.

Summary. The gonadotrophic potency of pituitary glands from crossbred Lincoln × Rambouillet ewes was significantly higher than that of pituitaries from purebred Hampshire ewes. The gonadotrophic potency of glands from both breeds increased markedly during a 90-day period following spaying whether during the breeding season or during anestrus.

15661

Utilization of Glucose and Acetone Bodies by Gastrointestinal Tract in Fasting Normal and Diabetic Dogs.

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Although the method of arterio-venous differences has often been used to study the metabolism of muscle[1-5] and of brain[6,7] in both normal and diabetic animals and men, this procedure has apparently seldom been applied to the gastrointestinal tract. It is now generally recognized that the RQ of the brain is always close to unity, and that it continues to remove glucose from the blood and utilize this sugar as its chief, if not its only fuel, under a great variety of conditions including fasting and diabetes. In skeletal muscle, however, it has been found by all who have studied A-V differences in severe diabetes that either no glucose is removed or the amount removed is greatly reduced even after the administration of this sugar. Only Cavett and Seljeskog[4] report that as much glucose is removed in diabetic patients as in normal controls, but it seems improbable that they studied severe diabetes for only one of their 8 patients had a blood sugar level above 180 mg/ml. Others have also reported nearly normal values in mild diabetes,[1,3] but absent or reduced A-V differences in severely diabetic patients.

London[8] found that the portal blood of normal dogs contained 6 to 9 mg/100 ml less glucose than the arterial, and that lactic acid might be either removed or added. Cherry and Crandall,[9] in dogs fasted 18 hours, observed a difference of 2.9 mg of glucose and no significant change in lactic acid. In amytalized dogs fasted 28 hours, Wierzuchowski and Fiszel[10] report the removal of 1.9 mg of glucose and the addition of 3.1 mg of lactic acid.

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In the course of determinations on hepatic glucose output and blood flow in normal[11] and diabetic[12] dogs, an opportunity was presented to obtain data on the retention or output of glucose, lactic acid and acetone bodies by the gastrointestinal tract.

Methods. London cannulae were applied to the portal (and hepatic) veins of normal dogs by the usual method. Other animals were similarly angiostomized and the pancreas completely removed at the second stage of the operation. Survival of the angiostomized-depancreatized dogs was promoted by postoperative treatment with sulfathiazole. Both normal and depancreatized animals were trained until they were not disturbed by the procedure of withdrawing blood. The diabetic dogs were kept on a diet of beef heart, raw pancreas, and milk with sufficient insulin (regular and protamine separately injected once daily) to reduce the urinary glucose loss to 1 or 2 g per day; these animals were not used until they had been stable on this regime for at least 2 weeks. The normal dogs were fasted 3 to 5 days. Blood samples were then taken from the femoral artery and portal vein at intervals of 1 or 2 hours until 3 or 4 pairs of samples had been removed. Similar pairs of samples were taken from the depancreatized dogs 3 to 7 days after withdrawal of food and insulin. All blood samples were analyzed for glucose by the method of Shaffer and Somogyi,[13] lactic acid by the method of Barker and Summerson,[14] and acetone bodies by the method of Crandall.[15]

Results. In Tables I and II we present the data on arterio-portal differences in normal dogs and in depancreatized dogs that have been without food and insulin for 3 or more days. Since blood sugar levels and urine glucose loss in the depancreatized dogs became stabilized on the third day, we consider that the latter animals were completely diabetic at this time.

It will be noted that the gastrointestinal tract of the normal dog removes significant amounts of glucose and acetone bodies from the arterial blood, but has no consistent effect upon the concentration of lactic acid. In more than half of all pairs of samples, there was no appreciable effect on lactic acid concentration, and although lactic acid was removed more often than it was added, the behavior of the gastrointestinal tract toward this substance is unpredictable. These statements apply equally to animals fasted 3 and 5 days.

There are suggestive differences with respect to glucose and acetone bodies between dogs fasted 3 days and those fasted 5 days as shown in Table III, although none of these differences is statistically significant.

The utilization of glucose, in terms of quantity per unit blood flow, is seen to be 1.6 mg/100 ml higher in the diabetic dogs than in normal animals. This is an increase of 90% over the normal value, but the difference only approaches statistical significance; $t = 2.1$, $P < 0.05$. In the diabetic dog there is also an appreciable utilization of lactic acid, which is probably significant, and the utilization of acetone bodies is 1.4 mg/100 ml greater than in normal dogs. The "t" for the difference in acetone utilization between normal and diabetic dogs is 3.1, $P < 0.001$. The average blood levels in

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[9] Cherry, I. S., and Crandall, L. A., Jr., *Am. J. Physiol.*, 1938, **125**, 41.

[10] Wierzuchowski, M., and Fiszel, H., *Bioch. Z.*, 1935, **282**, 124.

[11] Lipscomb, A., and Crandall, L. A., Jr., in press.

[12] Crandall, L. A., Jr., and Lipscomb, A., in press.

[13] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

[14] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[15] Crandall, L. A., Jr., *J. Biol. Chem.*, 1940, **133**, 539.

TABLE I.
Normal Dogs.
Arterio-portal Differences of Glucose, Lactic Acid, and Acetone Bodies.

	Mean A-P differences and standard errors		
	Glucose*	Lactic acid*	Acetone bodies*
Total No. of samples	57	38	37
Mean value, mg/100 ml	+1.8 ± 0.3	—0.1 ± 0.22	+0.38 ± 0.11
G-I removal: No. samples†	30	6	14
Range‡	+2 to +6	+0.6 to +5.4	+0.6 to +1.8
No change: No. samples†	23	20	21
Range‡	+1 to —1	+0.5 to —0.5	+0.5 to —0.5
G-I additions: No. samples†	4	13	2
Range‡	—2 to —5	—0.6 to —2.5	—0.6 to —0.9

* + indicates removal by gastrointestinal tract, — addition to blood.

† No. samples indicates the number of times that an amount of substance beyond the limits of error of the chemical method was removed or added, all others considered as "no change."

‡ "Range" indicates the range of value taken as indicating removal, no change, or addition, respectively.

TABLE II.
Diabetic Dogs.
Arterio-portal Differences of Glucose, Lactic Acid, and Acetone Bodies.

	Mean A-P differences and standard errors.		
	Glucose*	Lactic acid*	Acetone bodies*
Total No. of samples	33	33	33
Mean value, mg/100 ml	+3.2 ± 0.6	—0.37 ± 0.16	+1.8 ± 0.45
G-I removal: No. samples†	21	4	25
Range‡	+2 to +10	+0.6 to +2.5	+1.1 to +7.8
No change: No. samples†	8	15	5
Range‡	+1 to —1	+0.5 to —0.5	+1.0 to —1.0
G-I additions: No. samples†	4	14	3
Range‡	—2 to —3	—0.6 to —2.5	—1.1 to —5.3

*, †, ‡ as in Table I. Note that the range considered to be within the limits of error of the acetone body method is 1.0 mg/100 ml in diabetic dogs as compared with +0.5 in normal dogs; the higher range for the depancreatized animals is due to their higher blood acetone body level.

TABLE III.
Comparison of Arterial Blood Levels and Arterio-portal Differences in Dogs Fasted 3 and 5 Days.
(None of these changes is statistically significant.)

	Glucose		Lactic acid		Acetone bodies	
	Arterial blood conc.	A-P diff.	Arterial blood conc.	A-P diff.	Arterial blood conc.	A.P. diff.
3 day fasted	69	1.9	8.3	+0.2	2.9	0.23
5 " "	72	1.3	8.5	—0.35	5.6	0.53

the diabetic dogs were: glucose, —336, lactic acid, —13.4, and acetone bodies —67.9. The blood sugar and lactate levels showed a tendency to increase somewhat on the fifth, sixth, and seventh days after withdrawal of food and insulin, although no statistically valid correlation can be made. The acetone body concentration averaged 37.6 on the third day and 86.3 on the fourth or later days, the highest value attained being

210 mg/100 ml in one dog on the fourth day.

Discussion. From the data presented above we may conclude that the gastrointestinal tract of the fasting normal dog utilizes glucose and acetone bodies, but has no consistent effect on the concentration of lactic acid. It should be noted that removal of appreciable quantities of acetone bodies could not be expected to occur until the animal had been without food for at least 24 hours, for in the postabsorptive state these substances are not being manufactured by the liver and their concentration in the blood is insignificant.[16] We may also conclude that the gastrointestinal tract of the depancreatized dog from which food and insulin have been withheld for at least 3 days: (1) removes glucose possibly at a greater rate than the G-I tract of the normal animal, (2) adds lactic acid to the blood, and (3) removes acetone bodies at a more rapid rate than is true of the normal dog, the difference being in line with the higher blood acetone body concentration of this preparation.

Our present observations on normal dogs confirm the removal of glucose and inconsistent effect on lactic acid previously noted in normal unanesthetized dogs,[8,9] although the quantity of glucose taken up is less than reported by London.[8] The uptake of lactic acid reported by Wierzuschowski and Fiszel[10] may be attributed to their use of amytal anesthesia, since it has been shown that anesthetics may alter metabolic processes.[17] Utilization of acetone bodies by the normal gastrointestinal tract and the A-P differences in depancreatized dogs do not appear to have been reported previously. The structures drained by the portal vein may now be ranked with skeletal muscle in their ability to use acetone bodies, and brain remains the only tissue which has been shown not to remove them.[18]

Of particular interest is the continued utilization of glucose by the gastrointestinal tract in the diabetic dog. Assuming that the portal blood flow is 1.5 liters/kg/hour, our value would indicate the removal of 48 mg of glucose/kg/hour. Of this quantity some 11.6% is returned to the blood as lactic acid, leaving about 42 mg/kg/hour unaccounted for and presumably oxidized. This quantity would yield nearly 4 calories per kg per day. It has been shown that brain utilizes little, if any, other fuel than glucose in diabetes and accounts for about 10% of the total resting metabolism.[7] If then the utilization of glucose by the gastrointestinal tract accounts for another 8%, approximately 18% of the heat production of the resting diabetic dogs could be accounted for by the oxidation of glucose.

This inference, that 88% of the glucose removed by the gastrointestinal tract is oxidized, must of course be regarded as tentative, pending the demonstration of the actual fate of this sugar. However, it is difficult for us to conceive of any other explanation for its disappearance.

Summary. In the normal dog fasted 3 or 5 days, the organs drained by the portal vein remove significant amounts of glucose and acetone bodies, but have no consistent effect upon the blood lactic acid. In the depancreatized dog, 3 to 7 days after the withdrawal of food and insulin, these organs continue to remove statistically significant quantities of glucose and increase their removal of acetone bodies. Under these conditions they add lactic acid to the blood. If the glucose removed by the gastrointestinal tract of the depancreatized dog and not returned to the blood as lactate is oxidized, it will account for approximately 8% of the animal's resting metabolism.

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[16] Crandall, L. A., Jr., Ivy, H. B., and Ehni, G. J., *Am. J. Physiol.*, 1940, **131**, 10.

[17] Cherry, I. S., and Crandall, L. A., Jr., *Am. J. Physiol.*, 1937, **120**, 52.

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15662

Acetylation of Sulfanilamide as Influenced by the Thyroid.*

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The acetylation of sulfanilamide has proved to be a useful reaction for the determination of the acetylating capacity of the animal organism. The reaction has been shown to take place in the liver[1,2] in most animal species and to be irreversible.[3-5] An increase in the degree of acetylation was observed following the administration of various metabolites, such as acetate,[6-9] pyruvate,[7,10] lactate,[7] alcohol[6] and glucose.[7] The *in vitro* experiments of Klein and Harris[2] and of Lipmann[11] demonstrated an increased reactivity if acetate, pyruvate, lactate, acetoacetate, or acetoin were added to the reaction vessels. Lipmann, using pigeon liver as the most adaptable tissue for these studies, found the conjugation to take place not only in the slice but also in homogenates and even in extracts of the liver.

Inasmuch as the thyroid greatly affects metabolism and products of intermediary metabolism have been shown to influence the degree of acetylation of administered aromatic amines, a study of the effect of the thyroid on the *in vivo* acetylation of sulfanilamide was undertaken.

Methods and Results. The experiments here reported were carried out on female rats weighing approximately 200 g. The series consisted of untreated controls, rats which had received a diet consisting of 1% desiccated thyroid for 2 to 3 weeks, rats which had been thyroidectomized 2 to 3 months prior to the experiment, and rats which had received 0.1% thiouracil in their drinking water for 3 weeks. The animals were fasted overnight, injected with 5 ml of approximately 1% sulfanilamide solution, put in metabolism cages with free access to food and water for 24 hours, and the urine collected and analyzed according to the method of Bratton and Marshall.[12] The total amount of sulfanilamide excreted was approximately the same in all instances. The per cent of this amount which was acetylated varied with the experimental conditions; these data are recorded in Table I.

Statistical analyses[13] of the data were carried out by the standard procedure for small samples of determining the standard error of the difference between the 2 means. In the experiments using hyperthyroid rats, the difference between the means of the control and experimental groups was more than 3 times the standard error, the difference between the means of the controls and thiouracil-treated group was 2.5 and between the controls and throidectomized group it was 2.2. Taken in conjunction with the probability of the occurrence of the deviations derived from Fisher's "t" table, which is shown in Table I, the difference found in the hyperthyroid group appears to be definitely significant; the differences in the thyroid-

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Stewart, J. D., Rourke, G. M., and Allen, J. G., *Surgery*, 1939, **5**, 232.

[2] Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, 1938, **124**, 613.

[3] Fishman, W., and Cohn, M., *J. Biol. Chem.*, 1943, **148**, 619.

[4] van Winkle, W., and Cutting, W. C., *J. Pharm. and Exp. Therap.*, 1940, **69**, 40.

[5] Robinson, E. J., and Crossley, M. L., *Arch. Biochem.*, 1943, **1**, 415.

[6] Bernhard K., *Z. physiol. Chem.*, 1940, **267**, 91, 99; 1941, **271**, 208; 1942, **273**, 31.

[7] Harrow, B., Mazur, A., Borek, E., and Sherwin, C. P., *J. Biol. Chem.*, 1934, **105**, xxxiv.

[8] Harrow, B., Mazur, A., and Sherwin, C. P., *Biochem. Z.*, 1937, **293**, 302.

[9] James, G. O., *Biochem. J.*, 1939, **33**, 1688; 1940, **34**, 633.

[10] Martin, G. J., and Rennebaum, E. H., *J. Biol. Chem.*, 1943, **151**, 417.

[11] Lipmann, F., *J. Biol. Chem.*, 1945, **160**, 173.

[12] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

[13] Arkin, H., and Colton, R. R., *An Outline of Statistical Methods*, 4th edition, 1939, pp. 126-127.

TABLE I.
Acetylation of Sulfanilamide.

Exp. condition	% acetylated: Exp.	% acetylated: Control[*]	Diff.	P[‡]
Hyperthyroidism	30 ± 1.8 (11)[†]	39 ± 1.6 (11)	—23%	.01
Thyroidectomy	33 ± 2.2 (7)	39 ± 1.2 (6)	—15%	.05
Thiouracil treatment	44 ± 1.5 (11)	39 ± 1.4 (11)	—13%	.05

[*] For each experimental animal a control animal was run at the same time to minimize differences due to temperature, humidity, diet, etc.

[†] Figures represent mean values ± the standard error. Numbers in parentheses refer to number of rats per group.

[‡] Probability of occurrence of deviation, obtain from Fisher "t" table.

ectomized- and thiouracil-treated groups just barely significant.

The results show that both hyperthroidism and thyroidectomy produced a decrease in the degree of acetylation of sulfanilamide, whereas thiouracil treatment caused an increase. It is difficult to interpret the variance in the effects of thyroidectomy and thiouracil. A possible explanation may be that thiouracil exerts other effects than those depressing the activity of the thyroid.

It may be noteworthy to compare these results with the changes observed in an entirely different experiment, in which the high energy containing phosphate compounds of the liver were determined under similar conditions;[14] hyperthyroidism was associated with a decrease in the specific activity of the labile phosphorus, thyroidectomy with no change in this value, and thiouracil treatment with an increased specific activity of the labile phosphorus.

Summary. The influence of the thyroid on the acetylation of sulfanilamide was studied. Hyperthyroidism and thyroidectomy caused a decrease in the amount of sulfanilamide acetylated; thiouracil treatment led to an increase.

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[14] Greenberg, D. M., Fraenkel-Conrat, J., and Glendening, M. B., unpublished observations.

15663

Effect of Penicillin on Blood Coagulation.[*]

Jessica H. Lewis. (Introduced by F. H. L. Taylor.)

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Moldavsky, Hasselbrook and Caterno[1] have recently reported that both oral and intramuscular penicillin consistently shorten the coagulation time of normal blood and produce a nonretractile clot. These authors also observed decreased bleeding times in patients receiving penicillin. Hines and Kessler[2] demonstrated increased sensitivity to heparin in 2 of 10 cases receiving penicillin but found no changes in the platelet counts or prothrombin times.

The widespread use of penicillin makes this an important clinical problem. Therefore, it was decided to investigate more thor-

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[*] The expenses of this investigation were defrayed in part by a gift to Harvard University from Smith, Kline and French Laboratories, of Philadelphia, and in part by a grant "in recognition of Dr. Francis W. Peabody's services" to the Foundation by the Ella Sachs Plotz Foundation.

[1] Moldavsky, L. F., Hasselbrook, W. B., and Caterno, C., *Science*, 1945, **102**, 38.

[2] Hines, L. E., and Kessler, D. L., *J. A. M. A.*, 1945, **128**, 794.

TABLE I.
In vitro Effect of Penicillin on the Coagulation Time of Normal and Hemophiliac Whole Blood.

Final conc. of penicillin (units) per 2 cc of blood	Coagulation time (min)	
	Normal	Hemophiliac
1000	6.5	33
250	6.5	32
25	6.5	33
5	8	30
2.5	8	30
1.25	8	32
0.75	7.5	32
0.5	7.5	32
0.25	7.5	32
0	7	30

Clot retraction normal in all tubes.

oughly the effects of penicillin on blood coagulation in both normal and hemophiliac subjects.

Methods and Material. Six normal and 6 hemophiliac subjects were studied: whole blood coagulation times by a modification[3] of the method of Lee and White at 37°C, prothrombin times by a modification[4] of the Quick Method[5] and clot retraction were determined repeatedly in all patients. Capillary bleeding times, platelet counts and plasma fibrinogen concentrations were determined in many of the experiments. Penicillin levels[6] were determined in all patients.

In vitro tests were performed to determine the effect of penicillin at various concentrations on the coagulation time of normal and hemophiliac blood. Two cc of whole blood was added to 0.1 cc of various 0.85% sodium chloride dilutions of penicillin and coagulation times measured.

Two normal and 2 hemophiliac subjects received oral penicillin in an initial dosage of 300,000 units, followed by 100,000 every 2 hours for 48 hours. Four normal and 4 hemophiliac patients received intramuscular sodium penicillin in single doses of 25,000, 50,000, 100,000 and 200,000 units each.

Results. In vitro: No effect was produced on the coagulation time of normal or hemophilic blood by the *in vitro* addition of various dilutions of penicillin. The results are shown in Table I.

In vivo: 1. Oral penicillin. No effect on the blood coagulation mechanism was observed after oral administration of penicillin in either normal or hemophiliac subjects. One typical case, a hemophiliac, is presented in Table II.

2. Intramuscular penicillin. Four normal and 4 hemophiliac patients received intramuscular penicillin in a single dose of 25,000, 50,000, 100,000 or 200,000 units without effect on coagulation time, clot retraction, prothrombin time, platelet count, bleeding time or fibrinogen concentration in spite of high plasma penicillin levels.

One normal and one hemophiliac subject, each of whom received 200,000 units of penicillin intramuscularly are presented in Tables III and IV.

Discussion. Penicillin is used with such frequency that any contraindication to its use should be thoroughly investigated. The suggestion that penicillin might alter the blood coagulation reaction to such an extent that intravascular coagulation could be produced or increased is important particularly in view of the frequency of penicillin ad-

TABLE II.
Effect of Oral Penicillin (100,000 Units Every 2 Hours) on the Blood Coagulation of a Hemophiliac Subject.

Time	Coagulation time, (min)	Clot retraction	Prothrombin time, (sec)	Fibrinogen, mg/100 cc	Penicillin level, units/cc
Before penicillin	150	normal	21	383	0
24 hr of penicillin	160	”	22	371	0.11
48 ” ” ”	160	”	22	338	0.22

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[3] Pohle, F. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1937, **16**, 741.

[4] Souter, A. W., and Kark, R. M., *Am. J. Med. Sci.*, 1940, **200**, 603.

[5] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sci.*, 1935, **190**, 501.

[6] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE III.
Effect on Blood Coagulation of 200,000 Units of Penicillin Intramuscularly in a Normal Subject.

Time (hr after injection)	Coagulation time (min)	Prothrombin time (sec)	Clot retraction	Bleeding time (min)	Fibrinogen, mg/100 cc	Penicillin, unit/cc
Initial	8½	22	normal	1	243	0
½	9	19	,,	1	236	7.15
2	7½	21	,,	1¼	232	0.45
3	7½	22	,,	1¼	204	0.11
4	8	22	,,	1	231	0.05

TABLE IV.
Effect on Blood Coagulation of 200,000 Units of Penicillin Intramuscularly in a Hemophiliac Subject.

Time (hr after injection)	Coagulation time (min)	Prothrombin time (sec)	Clot retraction	Bleeding time (min)	Platelet count, × 1000	Fibrinogen, mg/100 cc	Penicillin, unit/cc
Control	79	20	normal	1½	252	224	0
1	110	20	,,	1¾	289	339	7.15
2	80	20	,,	2¼	250	336	0.90
3	97	19	,,	2¼	258	256	0.11
4	105	21	,,	2	262	286	0.05

ministration to patients suffering from thrombophlebitis, coronary artery disease and bacterial endocarditis. We were unable to demonstrate any effect of penicillin on either the normal or hemophiliac blood coagulation reaction, and therefore do not feel that consideration of intravascular blood coagulation offers any contraindication to the clinical use of penicillin.

Conclusions. We were able to demonstrate any *in vitro* or *in vivo* effect of penicillin on the blood coagulation reaction. No significant changes were observed in coagulation time, clot retraction, prothrombin concentration, fibrinogen concentration, platelet count or bleeding time of normal or hemophiliac subjects.

We are indebted to Clare Wilcox for the penicillin determination.

15664 P

Studies of the Thoracic Duct Lymph in Experimental Liver Injury in Dogs.

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The purpose of the present communication is to report comparative studies of the bilirubin, alkaline phosphatase, cholesterol, bile acids and intravenously-administered bromsulfalein content of the blood and thoracic duct lymph of dogs acutely poisoned with carbon tetrachloride or manganous chloride. A number of investigators[1-3] have used carbon tetrachloride by stomach tube to produce liver injury. A marked increase in its toxicity can be obtained by simultaneous administration of ethyl alcohol.[4] Manganous chloride was shown by Findlay[5] to

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[1] Meyer, J. R., and Pessoa, B. S., *Am. J. Trop. Med.*, 1923, **3**, 177.

[2] Lamson, P. D., and McLean, A. J., *J. Pharm. and Exp. Therap.*, 1923, **21**, 237.

[3] Freeman, S., Chen, Y. P., and Ivy, A. C., *J. Biol. Chem.*, 1938, **124**, 79.

[4] Lamson, D. P., Gardner, G. H., Gustafson, R. K., Maire, E. D., McLean, A. J., and Wells, H. S., *J. Pharm. and Exp. Therap.*, 1923, **22**, 215.

[5] Findlay, G. M., *Brit. J. Exp. Path.*, 1924, **5**, 92.

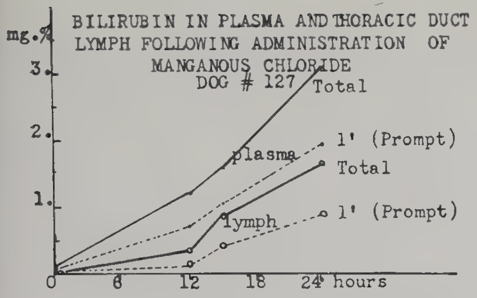

FIG. 1.

be toxic to the liver of rabbits, rats, and guinea pigs.

Material and Methods. Seven male dogs were used, ranging from 18 to 20 kg in weight. The anesthetic employed was pentobarbital sodium in an amount of 35 mg per kg of body weight. The thoracic duct lymph was secured in the following manner: In accordance with the Markowitz technic[6] the left subclavius vein was ligated, as were also the veins entering the external jugular just above its junction with the subclavius. Instead of ligating the innominate vein, as recommended by Markowitz, a string was placed around it just below the entrance of the thoracic duct. The left external jugular was then ligated and cut at a distance of 6-7 cm above the clavicle. A plastic cannula 3 cm in length* was introduced in the proximal cut end of the external jugular vein. A sample of thoracic duct lymph could then be secured by pulling the string upward, thus impeding the flow of lymph into the general circulation. At other times, the lymph flows through its natural channel, the string being loose and a small cork stoppering the cannula.

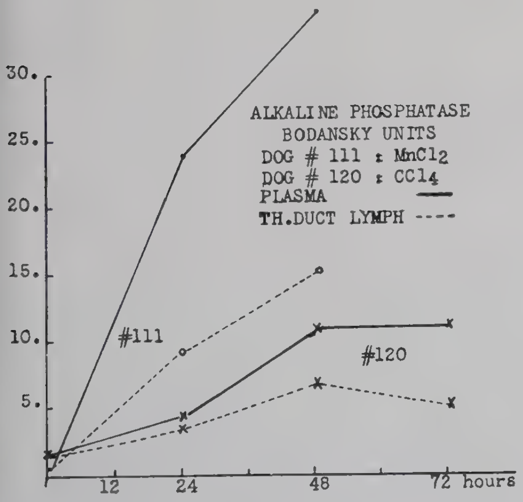

FIG. 2.

Four of the dogs were given 5 cc each of carbon tetrachloride, together with 5 cc of 95% ethyl alcohol by stomach tube immediately following thoracic duct cannulation and again after 24 hours. Each of the remaining 3 dogs were given 800 mg of $MnCl_2$ dissolved in 20 cc of physiological saline. Twenty-four hours later a second dose of 400 mg was injected into each of 3 dogs. These amounts were injected intramuscularly.

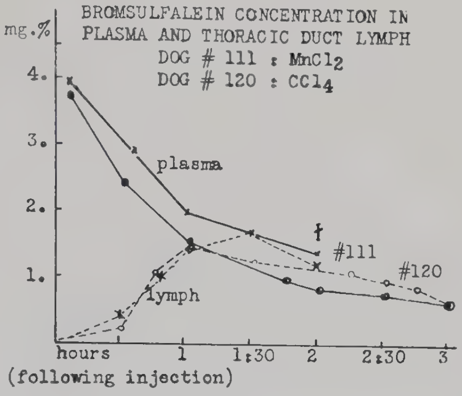

FIG. 3.

The laboratory methods used were the same as those described in a previous communication,[7] except for the alkaline phosphatase which was determined by the Bodansky method.[8]

Results. The bilirubin increased in both blood and lymph 12 hours following the first dose of $MnCl_2$ and 24 hours following the first dose of CCl_4. The main component of the blood and lymph bilirubin was the 1′ or prompt reacting type.[9,10] Fig. 1, from one of the $MnCl_2$ dogs is a typical example. The alkaline phosphatase increased as early as 12 hours following the first dose of $MnCl_2$ in both lymph and blood, and a definite increase was noted in all of the dogs 24 hours following the administration of either of the toxic substances. Fig. 2 compares the typical results from $MnCl_2$ and CCl_4 (one dog for each).

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[6] Markowitz, J., *Textbook of Experimental Surgery*, William Wood and Co., 1937.

* The author is indebted to Dr. J. H. Grindley, of the Mayo Clinic, Rochester, Minn., for suggesting the use of this type of cannula, and to the Irvington Varnish and Insulator Co., Irvington, N.J., for making it available.

[7] Gonzalez-Oddone, Miguel V., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 144.

[8] Bodansky, A., *J. Biol. Chem.*, 1937, **120**, 167.

The total cholesterol changed very little in any of the experiments, either in blood or lymph, if anything showing a slight tendency to decrease. The same might be said for the cholesterol esters. The bile acid concentrations in blood and lymph showed no significant alterations in the experiments with either of the 2 toxic substances. The behavior of the intravenously-injected bromsulfalein was similar in all 6 of the dogs in which it was administered (3 $MnCl_2$ and 3 CCl_4 dogs). As noted in Fig. 3, the bromsulfalein was retained in the blood at relative high concentration, a delayed increase appearing in the thoracic duct lymph similar to that previously described for the 32-hour stage following common duct ligation.

Discussion. The present results differ in a number of respects from those previously described for experimental obstructive jaundice.[7] The increase of serum bilirubin in the lymph was not as early nor as marked, and the same was true for bromsulfalein, although, as noted above, there was a delayed increase in the lymph, similar to that seen 32 hours following common duct ligation. The behavior of the bile acids was quite different in that, in the present experiments, no increase was noted, while in the experimental obstructive jaundice, the bile acids rose rapidly and to a marked degree in the lymph. The alkaline phosphatase behaved quite similarly in the 2 groups of experiments. While no attempt will be made to explain these observations, the possibility is considered that the differences are due to a relatively greater hepatocellular and lesser cholangiolar injury in the present experiments than in the experimental obstructive jaundice.

Summary. A method is described that allows collection of thoracic duct lymph over a period of days, the lymph circulating through its natural channel during the intervals in which it is not being collected. Liver injury caused by either CCl_4 or $MnCl_2$ were qualitatively comparable in dogs, the changes being more marked with the latter. An elevation of the alkaline phosphatase activity and bilirubin content of both blood and thoracic duct lymph were noted. The main component of the bilirubin was the 1′ or prompt reacting type.[9,10] The behavior of the injected bromsulfalein was comparable to that of the 32-hour stage, following common bile duct ligation.[6] No significant changes were observed as regards the bile acids, total cholesterol or cholester esters.

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[9] Ducci, H., and Watson, C. J., *J. Lab. and Clin. Med.*, 1945, **30**, 293.

[10] Watson, C. J., *Blood*, 1946, **1**, 99.

15665 P

Effect of Meals on the Electrocardiogram of Cardiac Patients.*

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In a previous communication,[1] we reported, for 12 normal men, the effect of meals on electrocardiograms recorded 30 and 60 minutes later. Most of the electrocardiographic functions, especially the T-wave, showed sta-

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* This work was supported by the Sugar Research Foundation, Inc., N.Y., and the National Dairy Council.

[1] Simonson, E., Alexander, H., Henschel, A., and Keys, A., *Am. Heart J.*, 1946, **32**, 202.

tistically significant changes, although the electrocardiograms remained within clinically normal limits. In 20 cardiac patients, mostly definite or suspected coronary disease, we recorded the standard limb leads, 3 chest leads (CF_1, CF_2, CF_4) and the heart sounds before and at 30 and 60 minutes after a 1000-calorie meal. In 2 patients additional chest leads were taken. The location for the chest leads was marked on the chest by means of a colored pencil.

Out of 10 patients with some symptoms of angina pectoris but with normal electrocardiogram before the meal and without any signs of decompensation, the electrocardiogram became abnormal after the meal. The abnormality involved inversion of the T-wave in lead CF_2 or CF_4 or increase in the size of a Q-wave in lead III. These patients felt no discomfort after the meal.

In 5 patients with coronary disease and abnormal electrocardiogram before the meal, the electrocardiogram became more abnormal with inversion of the T-wave in leads 1, 2 or CF_4. Slight changes of the QRS-contour were also observed. In one patient, a transitory *pulsus trigreminus* appeared after the meal.

In 2 patients with abnormal electrocardiograms before the meal the chest leads appeared to be more normal after the meal; previously inverted T-waves in CF_2 and CF_4 became positive. Similar changes have been observed after exercise in coronary patients and cannot be interpreted as a sign of improvement.

In 2 patients with coronary disease and abnormal electrocardiograms, the meal produced violent discomfort, associated with a pronounced ST-depression, especially in lead II. The discomfort as well as the ST-depression was instantaneously removed by nitroglycerine. In normal subjects the ST-segment does not change after the meal. It seems probable that the ST-depression is due to a transitory coronary insufficiency, produced by an abnormal reflex coronary constriction. In only one patient with an abnormal ECG were the changes after the meal comparable to those observed in normal subjects.

The present series is only of an exploratory nature, and no attempt is made to correlate the effect of meals on the electrocardiogram to the nature of the existing pathological changes. However, the series appears to show that the effect of meals is of importance for the clinical interpretation of electrocardiograms and for the standardization of procedure.

15666

Radiosodium Tracer Studies in Congestive Heart Failure.*

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Extensive studies in this laboratory on the distribution and turnover of sodium in cardiovascular states with Na^{24} (14.8 hour ½ life) as a tracer led to the possible investigation of long-term phenomena associated with congestive heart failure and edema. For such studies a long life isotope is necessary. Radioactive sodium 22 (3 year ½ life) is listed in the isotope tables but has not been reported as used in biological studies. Through the cooperation of Professors M. D. Kamen and A. L. Hughes of the Washington University Cyclotron, an experimental supply was made available for investigation. The details of preparation, standardization, determination and safety will be described later. In view of the significant results as well as for the introduction of this technic in the study of the mechanism of edema this report is presented.

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* Aided by grants from The Helis Institute for Medical Research, Mrs. E. J. Caire Fund for Cardiovascular Research, and The Life Insurance Medical Research Fund.

Two patients were followed simultaneously for 10 weeks. All voided and catheterized urine specimens and daily blood samples were collected during the first 3 weeks for the control and in the first 4 weeks for the heart failure patient. Samples of feces, vomitus, ascitic fluid and edema fluid were obtained when possible. Over 1500 5-minute counts were made. All samples were done in triplicate.

The congestive heart failure patient (elderly colored female) had hypertensive cardiovascular disease with acute failure and anasarca. This patient showed little if any improvement during her illness and died of acute heart failure 11 weeks after the study was started. The control was a young, colored female with vague arthraligic complaints of unknown cause. Both patients received the Na^{22} (less than 0.1 millicurie) as 200 cc of a 1% aqueous sodium chloride solution.

These patients were allowed to reach equilibrium with respect to the introduced sodium (more than 12 hours) before the results presented below were obtained. The determinations were made with a TA-Bl and scale-of-64. Technical Associates Geiger-Muller Counter.

Results. The effect of sodium intake on the excretion of sodium and water in the normal, and in congestive heart failure with and without the use of a mercurial diuretic (sodium salt of methoxyoximercuripropylsuccinylurea with theophylline,—"Mercuhydrin," 1 cc I.M.)[†] was studied. It was found that the control required 5 days to reach a steady state of sodium excretion when placed on a low sodium diet. The results summarized in Fig. 1 are based on the succeeding 4 days. The congestive heart failure patient was on a low sodium diet (1.7 g NaCl or less, daily) without a diuretic for 9 days. During this time the mean sodium output of the control was 3.1 times that of the abnormal. This is to be expected because the available sodium was reduced in both patients. However, when it became readily available, such as when they were on a regular hospital diet (10 g or more NaCl) with salt *ad lib.*, the difference became quite marked. During 6 days (without a diuretic) for the abnormal and 10 days for the control the latter excreted 90 times as much sodium. In one 24-hour period this exceeded 200 times as much. That this was not due to the difference in urine volume is indicated by the fact that the latter were essentially the same on a low sodium intake and only 3.5 times greater in the control on a regular intake.

The individual urine concentrations of Na^{22} for the control were quite variable and no regular relationship existed between urine volume and Na^{22} concentration. A 1 cc dose of the diuretic produced no appreciable effect in this patient. The congestive heart failure patient's urine concentrations of Na^{22} were fixed at a very low level and in many instances the counts were at background. There was little relationship to volume, *e.g.*, a 10-fold change in volume was accompanied by no concentration changes. It might be added that the 6-day normal salt intake period for the congestive heart failure patient was initiated by a slow infusion of 1000 cc of physiological sodium chloride solution.

The question of selective sodium excretion after a mercurial diuretic is conclusively answered for this patient. During the salt-free period (20 days) 10 1-cc injections of the diuretic were given intramuscularly. The average response was a 7-fold increase in salt output (Fig. 1). When salt was made available for 6 days this response consisted of an average increase of 75 times. In one 24-hour period it reached a 160-fold increase. Following the diuretic, the water output increased only 2.3 times with salt restriction and 5.3 times with salt given *ad lib.* The sodium diuresis preceded the water diuresis by 2-4 hours. The diuretic became effective in less than 2 hours, reached a maximum in 4-6 hours, and lasted rather uniformly for almost 24 hours when given intramuscularly.

The question next arose as to the degree of normalcy approached by the treated congestive heart failure patient as determined by a comparison with the control under

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[†] Courtesy Lakeside Laboratories, Milwaukee, Wis.

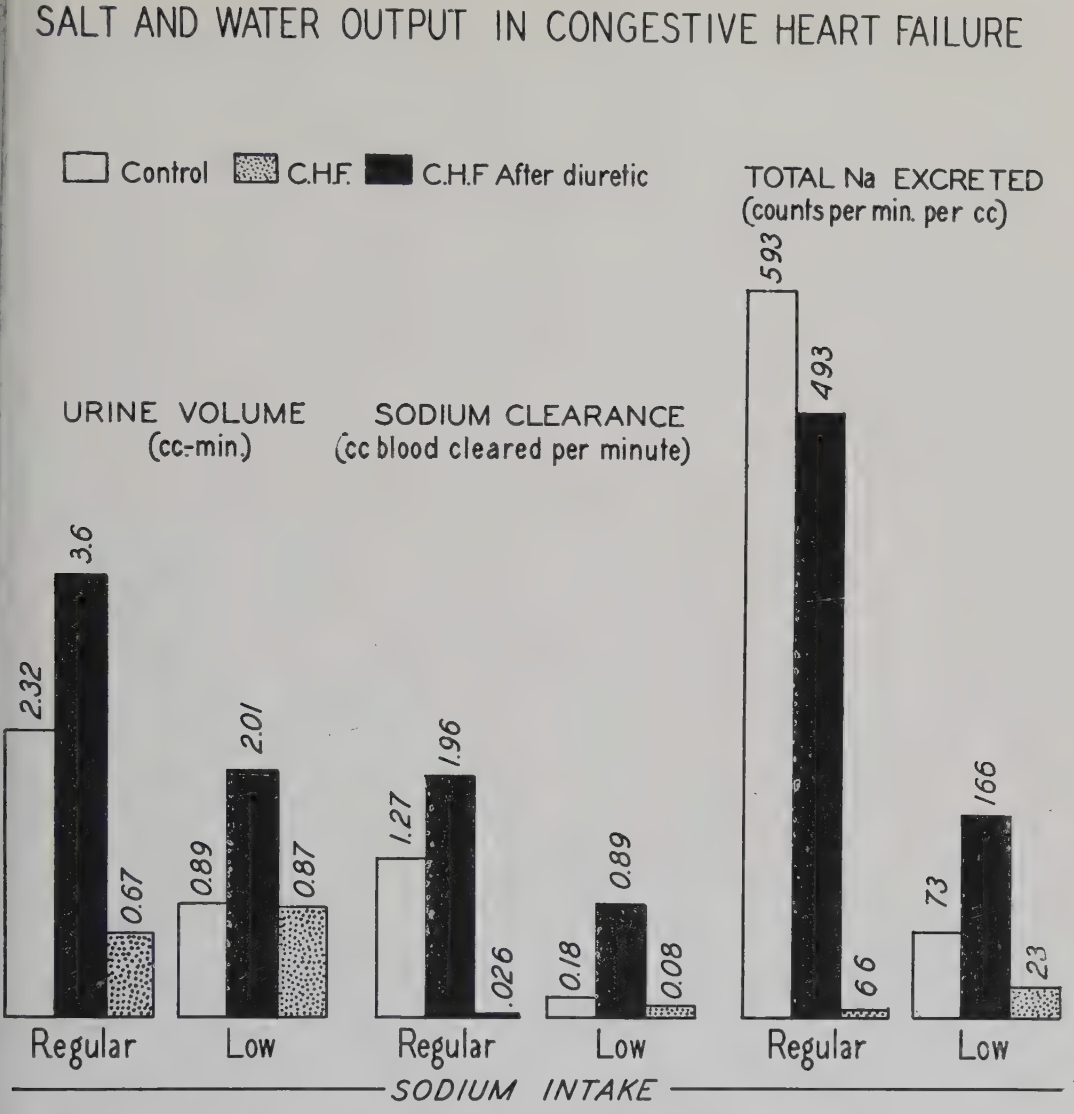

Fig. 1.

A comparison of the urine output, sodium clearance, and total sodium excretion on regular and low sodium diets in a congestive heart failure patient with and without a diuretic and a control subject. The heart failure patient was on a regular diet for 6 days and on a salt-free diet for 9 days, without a diuretic being administered, and 20 days during which 10 injections of a diuretic were given. The control subject was on a salt-free diet for 9 days (with only the last four used in the calculations) and on a regular diet 10 days.

identical conditions of salt and water intake. With mercurial diuresis the congestive heart failure patients' excretion of sodium was practically identical with the control and the excretion of water was greater (Fig. 1). In addition to the above data it was noted that: (a) The feces and vomitus counts were low and close to background. (b) The ascitic fluid and edema fluid were not appreciably different from the blood serum levels. (The removal of 1500 cc of ascitic fluid yielded as much sodium as would be excreted during 20 days without a diuretic). (c) In the control subject the blood serum Na^{22} concentration was found to decrease proportionately as the Na^{22} was excreted in the urine. In the congestive heart failure patient the presence of edema when the sodium was injected made similar calculations impossible. (d) The Na^{22} was 50% excreted by the control subject in 12 days and had disappeared in 10 weeks. In the congestive heart failure only 45% had been excreted in 10 weeks.

Discussion. The urine concentration of sodium is somewhat dependent upon the blood concentration at the time the urine is being formed. The exact relationship is unknown. Sodium clearances were employed to express this relationship. It must be remembered, however, that the term "clearance" cannot be used in the strict sense for sodium since the blood is less than 1% cleared of sodium during a single passage through the kidney. These patients formed urine of similar maximal concentration throughout the experiment. In the control this was equal to the blood concentration. In the congestive heart failure case it was lower but approached the blood level as the latter declined. It appears that the sodium concentrating ability in the congestive heart failure patient is fixed and not related to the blood level, whereas marked variations were possible in the normal.

Summary. The use of a radioactive tracer, Na^{22}, on an apparently normal control and a congestive heart failure patient showed a marked difference in respect to the behavior of this substance. (1) The control patient excreted an average of 90 times as much sodium as the congestive heart failure patient when both were given salt *ad lib.* (2) A mercurial diuretic resulted in an increase in sodium excretion of 75-fold when the congestive heart failure patient was receiving salt *ad lib.* (3) A reduction of salt excretion occurred in both when salt intake was restricted. However, the use of the diuretic still resulted in a 7-fold increase in the output of sodium in the congestive heart failure patient. (4) Other significant findings are presented.

The valuable technical assistance of Miss Shirley Wiederecht and Messrs. G. Morgavi, F. M. Parks, J. Fruthaler, and R. Schramel is gratefully acknowledged.

15667

Effect of Glycine Feeding on Renal Hemodynamics of the Rat.*

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The feeding of excess protein or its constituent amino acids has been found generally to increase the urea clearance in man[1,2] and in dog.[3,4] However, other than the studies of Van Slyke *et al.*,[3] little is known about the effect of these substances on the renal blood flow. Moreover, some investigators[5,6] have observed that the creatinine clearance did not rise after the ingestion of excess amino acids. It seemed important then, to study the effect of the common amino acid, glycine on the renal hemodynamics of the rat. This amino acid was employed because most investigators[2,4] are agreed that whatever effect protein does have on renal function, resides in its amino acid content which in turn almost invariably con-

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[*] Aided by a grant from the Dazian Foundation for Medical Research.

[1] Addis, T., and Drury, D. R., *J. Biol. Chem.*, 1923, **55**, 629.

[2] Longley, L. P., and Miller, M., *Am. J. Med. Sci.*, 1942, **203**, 253.

[3] Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A., *Am. J. Physiol.*, 1934, **110**, 387.

[4] Pitts, R. F., *J. Nutrition*, 1935, **9**, 657.

[5] Pitts, R. F., *Am. J. Physiol.*, 1944, **140**, 535.

[6] Beyer, K. H., Wright, L. D., Russo, H. F., Skeggs, H. R., and Patch, E. A., *Am. J. Physiol.*, 1946, **146**, 330.

ains glycine.

Methods. Seventeen male, adult albino ats, approximately 6 months of age and reviously subsisting on Purina Dog Chow ere used for this study. Creatinine and ippurate clearances were done to ascertain he control rates of glomerular filtration and enal plasma flow respectively. Approximately one week later, each rat received 0 mg of glycine per 100 g of body weight y stomach tube and the clearance studies ere repeated immediately afterwards. The methods and calculations used in determining the clearances have been reported in a previous study.[7] All clearances have been expressed in cc per hour per 100 g of body weight.

TABLE I.
The Effect of Glycine on Creatinine and Hippurate Clearance of the Rat.

	Before Glycine			After Glycine		
Rat	U.V.*	C.C.†	H.C.‡	U.V.	C.C.	H.C.
27	1.75	30.4	180	2.9	41.5	101
25	3.60	40.8	212	4.4	36.2	149
93	4.95	39.0	90	5.2	41.5	144
72	3.70	39.4	139	2.0	28.0	182
92	5.0	48.8	175	3.5	39.2	157
57	4.0	39.5	202	4.7	40.0	146
10	0.60	18.6	91	4.75	44.0	185
12	2.4	34.8	146	3.55	38.6	89
98	2.3	34.6	108	2.80	28.6	119
35	3.6	34.4	158	2.40	40.8	142
29	4.9	28.5	115	0.90	20.8	64
88	3.9	43.7	160	2.10	24.7	108
21	3.6	38.7	134	3.30	19.8	122
09	3.5	38.8	128	2.75	39.7	128
20	4.6	32.5	73	2.25	45.7	81
77	4.6	37.0	138	2.85	36.8	191
93	4.55	50.5	152	3.05	42.8	138
Av.	3.62	37.1	141	3.14	35.8	132

* U.V. equals urine volume in cc per hour.

† C.C. equals creatinine clearance in cc per 100 g per hr.

‡ H.C. equals hippurate clearance in cc per 100 g per hour.

Results. Inspection of Table I indicates that no significant change occurred in either the creatinine or hippurate clearance of the rat following the ingestion of glycine. Thus the average creatinine clearance was 37.1 cc per hour before and 35.8 cc per hour after the ingestion of glycine. Likewise, the average hippurate clearance was 141 cc per hour before and 132 cc per hour after glycine had been given. The average urine volume per hour was 3.62 cc before and 3.14 cc after ingestion of glycine. This average decrease of 13% is perhaps of questionable significance.

It should be noted that Pitts[5] also found no increase in the creatinine clearance of dogs after heavy glycine feeding, nor could Beyer *et al.*[6] observe an increase in the dog's creatinine clearance after feeding of such essential amino acids as tryptophane, leucine, isoleucine or valine. Finally it should be emphasized that the results of this present study only indicate that *excess* feeding of glycine appears to have no effect on renal hemodynamics. It is conceivable that were glycine to be reduced abnormally in the blood, changes in renal hemodynamics might occur.

Conclusion. The administration of glycine to the rat had no demonstrable effect on either its rate of glomerular filtration or its renal blood flow.

The author wishes to express his thanks to Barbara Trousdale and Frances Greenberg for their technical assistance.

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[7] Friedman, M., *Am. J. Physiol.*, 1947, to be published.

15668

Differentiation of Soy Bean Antitryptic Factors.

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In a preliminary report, the author called attention to a water-soluble antitryptic fraction of navy beans which can be readily precipitated with alcohol. Although a large part of the trypsin-inhibiting material of soy beans is not precipitated with alcohol, it is precipitated with acetone.[1] It was further pointed out that soy and other beans as well as navy beans do, however, contain a trypsin-inhibiting fraction which can be repeatedly reprecipitated with alcohol.[2]

Kunitz soon crystallized this latter factor from soy beans and described some of its properties.[3,4] He found it to be a crystalline globulin which can be further recrystallized from 20% alcohol. Further comment regarding the occurrence of this factor is hardly necessary; however, the trypsin-retarding fraction of soy beans, which is quite soluble in 60% alcohol but insoluble in acetone, would appear to deserve further attention. Following the publication of our description of this fraction,[1] it was found that a note by Ham and Sandstedt[5] had appeared shortly before referring to what appears to be this material, and we wish to acknowledge their paper. They indicated that this antitryptic substance is destroyed upon treating an aqueous extract of the beans with 60% alcohol, and they found no inhibiting action in aqueous extracts of soy beans previously treated with 45% alcohol. Other properties given also differentiate it from the antitryptic substance which is found in alcohol precipitates of aqueous extracts of soy beans. These investigators may have discarded the latter substance with the kaolin used to remove proteins since it will adsorb on kaolin.

A number of soy bean preparations having antitryptic properties have been used in various investigations. Employing an acetone precipitate of an aqueous extract, Ham, Sandstedt, and Mussehl pointed out that the proteolytic-inhibiting material has a retarding effect on the growth of chicks.[6] Klose, Hill and Fevold observed similar growth effects and indicated that the substance which they employed is nondialyzable, can be precipitated by salts, is inactivated by heat, and appears to be a protein which is concentrated in the acid-soluble (pH 4.2) fraction of soy bean protein.[7] Tagnon and Soulier, employing the inhibitor obtained by acetone precipitation of aqueous extracts as well as the crystalline material of Kunitz, observed marked anticoagulant activity on human whole blood and plasma.[8] Employing the acetone-insoluble and alcohol-insoluble fractions, the author has been unable to show a consistent influence on trypsin shock.[2] Thompson has indicated that the alcohol-insoluble factor of navy beans does not modify anaphylactic shock produced by egg albumin.[9]

Unless definite precautions are taken, crude soy bean antitryptic preparations may consist of varying proportions of at least 2 factors. This occurs especially when acetone is used to precipitate the active material from an aqueous extract. The object of the present report is to call attention to the difference between the soy bean antitryptic substance which can be extracted with water and precipitated with alcohol, called the alcohol-insoluble factor, and the active material which can be extracted with 60% alcohol and precipitated with acetone, called the acetone-insoluble factor. Both are soluble in water.

Methods. Relative inhibition of tryptic activity was estimated by adding the experimental material to triplicate casein digestion preparations, and the degree of digestion was compared with that of the same number of control uninhibited preparations of equal volume, run simultaneously. With final concentrations of 0.01% of crude trypsin and 3.1% casein, approximately half of the casein was digested at 38°C in one hour in the absence of inhibiting agents. The actual degree of digestion was determined by the increase in

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[1] Bowman, D. E., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 139.

[2] Bowman, D. E., *Fed. Proc.*, 1945, **4**, 84.

[3] Kunitz, M., *Science*, 1945, **101**, 668.

[4] Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

[5] Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

[6] Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

[7] Klose, A. A., Hill, B., and Fevold, H. L., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 10.

[8] Tagnon, H. J., and Soulier, J. P., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 440.

[9] Thompson, R. L., personal communication.

TABLE I.
Comparison of Antitryptic Preparations.

Treatment and portion used	% of normal uninhibited digestion in the presence of inhibiting substances		
	Acetone-insoluble fraction, 0.25%*	Alcohol-insoluble fraction, 0.04%*	Crystalline material, 0.04%*
Untreated	49.9	52.5	46.9
Material insoluble in 60% alcohol	103.0	54.6	47.3
Untreated	45.5	54.2	46.8
Material insoluble in a solution 40% saturated with ammonium sulfate	102.0	56.6	49.5
Untreated	44.8	51.0	47.0
Material soluble in 2.5% trichloracetic acid	46.0	97.0	99.4

* Concentration of untreated trypsin inhibiting fraction in the casein digestion mixtures. The concentration of the treated material would have been the same except for the influence of the treatment.

the refractive index[10] of the filtrate following the isoelectric precipitation of undigested casein with a constant amount of acetic acid. In all cases triplicate unincubated negative controls were run in which the casein was precipitated before the trypsin was added and the filtrate was employed immediately. Triplicate values ordinarily agreed quite well throughout.

The material referred to as the acetone-insoluble factor was prepared by first extracting 100 g of finely ground untreated soy beans with one liter of 60% ethyl alcohol. The active material was then precipitated from the centrifuged and filtered alcohol extract by adding 2 volumes of acetone. The precipitate was centrifuged down and immediately dried in a vacuum desiccator. The majority of such preparations were further redissolved and heated in 2.5% trichloracetic acid at 98°C for 5 minutes. After centrifuging down and discarding the precipitate the active substance was reprecipitated from the neutralized solution with 90% acetone.

The alcohol-insoluble factor was obtained by extracting 100 g of finely ground untreated soy beans with one liter of water overnight, after adjusting to pH 4. After centrifuging, the supernatant aqueous extract was precipitated by adding alcohol to 60% concentration. The precipitate was centrifuged and dried in a vacuum desiccator.

Results. Numerous preparations of the 2 factors were employed and compared with a crystalline preparation provided by Dr. Kunitz. Typical results are given in Table I. As may be expected from the methods of preparation, the solubilities of the 2 factors in 60% alcohol are quite different. This can be shown more objectively by comparing the trypsin-inhibiting capacity of a suitable weighed amount of the untreated substance with the inhibiting capacity of an equal amount after extraction with 100 parts of 60% alcohol by weight. The acetone-insoluble factor appears to completely dissolve, and no activity is apparent after centrifuging and drying any possible insoluble material and adding it to casein digestion mixtures. Under the same conditions the alcohol-insoluble factor and the crystalline material appeared to be insoluble and, as judged by trypsin-inhibiting capacity, essentially entirely recovered in the undissolved material.

The acetone-insoluble factor does not precipitate when present in 1% concentration in a solution 40% saturated with ammonium sulfate. After centrifuging down any slight amount of insoluble material present, the latter was redissolved and the pH adjusted to that of the digestion mixture. No antitryptic activity was observed. In contrast, under similar conditions the alcohol-insoluble factor and the crystalline material give a definite precipitate and, in each case, the trypsin-inhibiting capacity of the precipitate indicates essentially complete recovery.

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[10] Robertson, T. B., *J. Biol. Chem.*, 1912, **12**, 23.

Although crystalline soy bean antitrypsin is precipitated when heated in 2.5% trichloracetic acid as pointed out by Kunitz,[3] the acetone-insoluble factor can be almost quantitatively recovered from the filtrate of such a solution. This is indicated by the final antitryptic capacity retained after so treating a weighed amount of the material. After heating this inhibitor, in 2% concentration, in 2.5% trichloracetic acid at 98°C for 5 minutes only a very slight precipitate forms. After centrifuging and discarding this precipitate and neutralizing the supernatant solution, the active material can be almost completely recovered by precipitating with 90% acetone.

Upon treating the alcohol-insoluble factor or the crystalline material in a similar manner, an appreciable precipitate is seen in the trichloracetic acid solution and the amount of antitryptic substance obtained from the neutralized filtrate by precipitating with 90% alcohol is insignificant.

A typical preparation of the acetone-insoluble fraction was found to contain approximately 1% nitrogen and gave only faintly positive biuret and Millon tests. Further observations dealing with the nature of the active material are in progress. Its solubility characteristics and a phosphorus content of about 5% in the present preparations suggests that some attention be given to phosphorus-containing substances.

Summary. In a number of respects, the trypsin-inhibiting factor of soy beans which may be extracted with 60% alcohol and precipitated with acetone differs from the antitrypsin which can be extracted with water and precipitated with 60% alcohol. These substances may be differentiated on the basis of their solubilities in a solution 40% saturated with ammonium sulfate, in 2.5% trichloracetic acid as well as in alcohol.

15669

Pharmacological Protection Against *Salmonella* Endotoxin and Certain Other Poisons.

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In previous work sulfonamides were found effective to a limited degree in counteracting the systemic toxicity of certain typical endotoxins of Gram-negative bacteria.[1] Analysis of this action of sulfonamides has led to the conclusion, reported here, that the efficacy of sulfonamides in this respect depends on their goitrogenic activity, and posed again the problem of devising an efficient therapy directed against this class of poisons.

The endotoxin-containing somatic (O) antigens of Gram-negative bacteria are frequently the principal source of damage in infections by these organisms.[2] In contrast to the classical bacterial exotoxins, antibodies for these somatic antigens have little antitoxic potency.[3] The action of these endotoxins is usually aborted by checking the multiplication of the bacteria either by means of previous immunization with appropriate vaccines or, when the infection has gained a foothold, by administration of antibiotics such as streptomycin. Although these measures are often dramatically effective when directed against the primary infection, they afford little or no protection against the intoxication ensuing once the bacteria have liberated their antigens into the bloodstream. Indeed the toxicity of *Shigella*[4] and *Salmonella*[5] vaccines employed for prophylactic immunization has been a complicating factor; and the excessive toxicity of *Shigella* vaccines has constituted a hazardous accompaniment to their successful use, even when neurotoxin-free vaccines are employed. It follows that the problem of therapeutics directed specifically at preformed endotoxin is of considerable clinical significance.

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[1] Hutner, S. H., and Zahl, Paul A., *Science*, 1942, **96**, 563; Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

[2] Zahl, Paul A., Starr, M. P., and Hutner, S. H., *Am. J. Hyg.*, 1945, **41**, 41.

[3] Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, 1944, **39**, 189.

The only pharmacological (as distinguished from immunological) agents known to lessen experimentally the toxic effects of these antigens were sulfonamide drugs, whose effectiveness, however, in this respect was low, protecting mice against only a few lethal doses of the endotoxin.

Accordingly, over several years we have undertaken a search for pharmacological agents which might be more effective than sulfanilamide in counteracting the effects of the endotoxin. We have tested the compounds and combinations of compounds listed below* for protective effects against the endotoxin in mice. The rationale governing their selection for trial need not be given here. It may be noted, however, that nearly

The tests in most instances consisted of injecting groups of 6 mice each with intraperitoneal doses of 2-LD_{50} of the endotoxin. Previously to or simultaneously with the injection of the endotoxin, the mice received by repeated intraperitoneal injections or by stomach tube, the test chemicals in sub-toxic doses. Survival of the mice at the end of 24 hours after injection of the endotoxin served as the index of reduction in toxicity brought about by the test chemicals.

The mice used in these tests and in those experiments presented below were all young males of a Swiss line in the weight range of 18-21 g, obtained from the Rockland Farms. The animals were maintained during the experimental period, unless otherwise specified on a commercial mouse diet and tap water *ad libitum*.

The methods used in preparing the endotoxin material from *Salmonella typhimurium* employed in the above tests have been previously described.[6]

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[4] Goebel, W. F., Perlman, E., and Binkley, F., *Science*, 1944, **99**, 412.

[5] Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, Williams & Wilkins, Baltimore, 3rd Ed., 1946, p. 1553.

* (1) N^1-acetylsulfanilamide; (2) N^4-acetylsulfanilamide; (3) *p*-aminoazobenzene; (4) *o*-aminobenzene sulfonic acid; (5) *p*-aminobenzoic acid; (6) anthranilic acid; (7) arginine; (8) arginine, cysteine, methionine; (9) ascorbic acid; (10) ascorbic acid, cortical adrenal extract; (11) ascorbic acid, guanine, thiamine, methionine; (12) ascorbic acid, cysteine, glycine, methionine, *p*-aminobenzoic acid, calcium gluconate, sodium acetate; (13) ascorbic acid, glutathione, (14) *p*-aminophenol; (15) atropine; (16) atropine, ergotoxin; (17) benzedrine; (18) benzenesulfonylamide; (19) bulbocapnine; (20) butanediol; (21) calcium gluconate; (22) carrot diet; (23) camphor; (24) catechol; (25) catechol (acetylated); (26) casein hydrolysate; (27) casein hydrolysate, ascorbic acid, adrenal cortical extract, sodium chloride; (28) choline; (29) coramine; (30) coramine, sulfanilamide, calcium gluconate; (31) coramine, atropine; (32) coramine, metrazol; (33) concanavalin; (34) cortical adrenal extract; (35) cortical adrenal extract, amino acid mixture; (36) cysteine; (37) cysteine, methionine; (38) cysteine, methionine, sodium acetate; (39) cysteine, glycine, yeast, nucleic acid, arginine, sodium acetate; (40) 4,4-diaminodiphenyl sulfone; (41) diaminophenol; (42) diacetyl; (43) diphenylsulfone; (44) ergotoxin; (45) glycolic acid; (46) glycine; (47) glutathione; (48) glutathione, monoethalolamine salt of ascorbic acid; (49) heparin; (50) hesperidine; (51) hesperidine, ascorbic acid; (52) histamine; (53) histamine, tryptophane, ascorbic acid, methionine; (54) histamine, tryptophane, methionine; (55) histidine; (56) hydroquinone; (57) *p*-hydroxybenzoic acid, hydroquinone triacetate, hydroquinone; (58) insulin; (59) insulin, dextrose; (60) liver (Difco); (61) liver (Lederle, 6E, 6C, 9C); (62) liver, sulfadiazine; (63) methionine; (64) metanilamide; (65) metrazol; (66) nicotinic acid; (67) *o*-nitrophenol; (68) *p*-nitrosophenol; (69) *p*-nitrobenzoic acid; (70) yeast nucleic acid; (71) nupercaine; (72) orcinol; (73) *o*-phenetidine; (74) *p*-phenetidine; (75) *o*-phenylenediamine; (76) picrotoxin; (77) quinone; (78) resorcinol; (79) sodium acetate; (80) sodium chloride; (81) sodium formaldehyde sulfoxylate; (82) sulfanilic acid; (83) thiamine; (84) thiouracil; (85) tryptophane; (86) tyrosine; (87) yeast, liver diet; (88) yeast extract (Difco).

[6] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 187.

TABLE I.

Dose of endotoxin (intraper.) mg	Dose of thiouracil (intraper.) mg	No. of animals	% survival
—	3.0 (×6)	10	100
0.50	3.0 ″	27	96
0.50	C*	31	78
0.75	3.0 ″	20	95
0.75	C	20	35
2.00	3.0 ″	24	60
2.00	C	25	12

* C = Control.

all these tests were conducted before the recognition of sulfonamides as goitrogenic agents. It suffices to say that the only non-sulfonamides counteracting to any observable degree the toxicity of the endotoxin were thiouracil[†] and *p*-aminobenzoic acid. The further experimental basis for these findings and certain collateral experiments are described here.

Procedure and Results. Protection by Thiouracil Against Endotoxin-induced Death. In a series of experiments involving a considerable number of mice, the animals were pretreated with thiouracil, administered intraperitoneally, for a period of 72 hours, by means of a 3-mg dose given about every 12 hours. The total dosage over 72 hours was thus 18 mg. Following the last thiouracil dose, lethal amounts of the endotoxin material[6] of *Salmonella typhimurium* were injected. Survival of the mice at the end of 24 hours was taken as a measure of the effectiveness of the thiouracil in lessening the toxicity of the endotoxin. Because of the general consistency of the results, the experiments are summated in Table I, rather than presented individually.

It is evident from the data listed in Table I that thiouracil affords a degree of protection against the toxic effects of the endotoxin. In other exploratory experiments in which only a few hours elapsed between the time of the first dose of thiouracil and the injection of the endotoxin, very little protective effect could be observed, due presumably to the slowness of interference with thyroid function by thiouracil. Also, when the pretreatment with thiouracil was extended to 7 days before the injection of the endotoxin, little if any protective effect was observed, due possibly to the general debilitating effects of sustained thiouracil treatment, as shown by the fact that mice so treated and with such relatively high dosages failed to gain weight at the normal rate.

Protection by Thiouracil Against Endotoxin-induced Tumor Hemorrhage. The toxicity of the endotoxins of Gram-negative bacteria is generally considered to result primarily from injury to the vascular endothelium. This action is readily studied experimentally by the induction of hemorrhage in transplanted tumors, as well as by the over-all lethality. We had previously found that the induction of tumor hemorrhage provides a sensitive, almost quantitative method for assaying small fractions of a lethal dose of endotoxin.[2] This belief that the hemorrhage-inducing property and the death-inducing property of the endotoxin are in fact low- and high-dosage manifestations of the same action, could undergo additional test by determining, using methods described elsewhere,[2] whether thiouracil could protect mice against endotoxin-induced tumor hemorrhage. Table II gives data indicating a positive tumor hemorrhage protective effect on the part of thiouracil.

Protective Effects of Thiouracil Against Other Poisons. A series of experiments was performed to determine whether thiouracil had any protective effects against liver poisons of the class typified by 1,2-dichloroethane. The effects of poisons of this class are physiologically quite different from those typified by the endotoxin. It seemed of interest, therefore, to ascertain whether a goitrogen such as thiouracil is effective against dichloroethane. The results of these experiments are summarized in Table III. It is concluded that thiouracil has some protective action against the toxicity of dichloroethane.

A similar type of experiment was performed with colchicine. This poison is presumed to

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[†] Kindly supplied by Dr. R. O. Roblin, Jr., of the American Cyanamide Company, Stamford, Conn.

TABLE II.

Dose of endotoxin (intraper.) mg	Dose of thiouracil (intraper.) mg	No. of animals	Degree of tumor hemorrhage*
0.10	3.0 (×6)	18	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 ++
0.10	(control)	14	+++ +++ +++ +++ ++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++ 0 0

* Each designation below indicates the degree of tumor hemorrhage induced in each mouse. 0 = no hem.; + = slight hem.; ++ = considerable hem.; +++ = marked hem.; ++++ = maximal hem.

TABLE III.

Dose of dichloroethane (in corn oil, by stomach tube) mg	Dose of colchicine (intraper.) mg	Dose of thiouracil (intraper.)	No. of mice	% survival
16	—	3.0 mg (×6)	81	53
16	—	(control)	66	18
—	0.50	3.0 mg (×6)	41	44
—	0.50	(control)	42	50

act primarily on the vascular endothelium, as does the endotoxin, and also produces a set of symptoms overtly similar to those produced by the endotoxin.[7] Therefore, testing colchicine against a goitrogen such as thiouracil seemed in order. The results are tabulated in Table III, and it is concluded that thiouracil has no protective effect against the toxicity of colchicine.

Effect of Temperature on the Toxicity of the Endotoxin. From other studies it has been concluded that the effects of thiouracil (on anoxia, etc.) are attributable to a general lowering of metabolic rate via interference with thyroid gland activity.[8] If such an action accounts for the thiouracil effect on the toxicity of the endotoxin and dichloroethane, then a similar effect on these poisons should be observed by altering general body metabolism in other ways. Accordingly, an experiment was set up in which groups of mice were kept at various temperatures for the 24-hour period following injection of the endotoxin. The results of this experiment are summarized in Table IV. Clearly, mice maintained at high environmental temperatures are more susceptible to the toxicity of the endotoxin than those kept at lower temperatures.

TABLE IV.

Air temperature of incubator, °C	Dose of endotoxin (intraper.)	No. of mice	% survival
14	0.75 mg	19	74
14	(control)	13	100
22	0.75 mg	20	35
22	(control)	20	100
30	0.75 mg	20	10
30	(control)	14	100

Effect of p-Aminobenzoic Acid on the Toxicity of the Endotoxin and of Colchicine. It has been reported that large doses of *p*-aminobenzoic acid have an action on the thyroid gland similar to that of thiouracil.[8] Experiments were performed to determine if this action would parallel that of thiouracil, when tested against the toxicity of the endotoxin and that of colchicine. The *p*-aminobenzoic acid was administered by stomach tube in 3 doses of 33 mg each; the first, one hour before injection of the toxic material, the other 2, at one-hour intervals after the injection. The results of these experiments are summarized in Table V. It is evident that *p*-aminobenzoic acid offers some protection

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[7] Ludford, R. J., *J. Nat. Cancer Inst.*, 1945, **6**, 89.

[8] Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Endocrinology*, 1945, **37**, 223.

TABLE V.

Dose of endotoxin (intraper.) mg	Dose of colchicine (intraper.) mg	Dose of p-aminobenzoic acid (stomach tube)	No. of mice	% survival
—	—	33 mg (×3)	15	100
0.75	—	33 mg (×3)	20	65
0.75	—	(control)	20	25
—	0.50	33 mg (×3)	15	0
—	0.50	(control)	15	14

against the effects of the endotoxin, but that, like thiouracil, it is ineffective against colchicine.

Discussion. The sulfonamides are now recognized as possessing a goitrogenic action.[9] Likewise the most evident physiological effects of thiouracil and of *p*-aminobenzoic acid are ascribable to interference with thyroid function.[8]

It seems reasonable to suppose, then, in view of the additional data presented in this paper, that the general protective effects of these 3 classes of compounds may be ascribed to a common mechanism, *viz.*, interference with thyroid function. This conclusion receives further support from some exploratory experiments in which mice for several days before endotoxin injection were fed on a diet containing 1% of thyroid extract. These animals appeared to be more susceptible to the toxic action of the endotoxin than were the untreated controls.

As to how thyroid interference and the presumed lowered metabolism renders the endotoxin less toxic, very little may be said. If, as has been postulated for colchicine,[10] the endotoxin is first oxidized to products which themselves are the effective poisons, then it is reasonable to suppose that the production of these secondary poisons would be retarded by a lowered metabolism, possibly allowing the normal excretory and detoxication systems of the body to cope with the antigen in its nontoxic phase.

The failure of colchicine to be affected by thiouracil treatment is provocative. There appears to be a general parallelism between the toxic effects of the endotoxin and the stereotyped response of the body to a considerable series of toxic agents. This body response has been termed "the general adaptation syndrome" by Selye,[11] and includes the diverse phenomena usually designated as "shock." It seemed appropriate to us to determine whether the action of goitrogens was specific for the endotoxin, or whether their effect might in an over-all way be directed at the harmful phases of "the general adaptation syndrome." Considerable interest was attached to colchicine in this connection, for colchicine poisoning induces a syndrome overtly resembling that of poisoning by the endotoxin, even to a parallelism in tumor hemorrhage induction.[7] Selye has stated that colchicine is an especially good reagent for eliciting the "alarm reaction"—the first stage of "the general adaptation syndrome." If one agrees that colchicine is indeed a typical elicitor of "the general adaptation syndrome," then goitrogen therapy in the later damaging phases of this syndrome is not likely to prove very useful, in view of the above reported failure of goitrogens to protect against colchicine. Some doubt, on the other hand, may be cast on the legitimacy of regarding colchicine as a reliable reagent for producing "the general adaptation syndrome" of Selye, especially in view of the protection obtained by goitrogens against dichloroethane poisoning, which bears little immediate resemblance to either colchicine or endotoxin poisoning. It may be remarked that protection against dichloroethane has also been reported for sulfanilamide and *p*-aminobenzoic acid, both of whose actions, like that of thiouracil, are goitrogenic.[12] Hence, it must be concluded that the influence of goitrogens on "the general adaptation syndrome" is an open issue.

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[9] Astwood, E. B., *J. Pharmacol.*, 1943, **78**, 79.

[10] Fuhrman, F. A., *Physiol. Rev.*, 1946, **25**, 247.

[11] Selye, H., *J. Allergy*, 1946, **17**, 231; *ibid.*, 1946, **17**, 289.

Penicillin has lately been reported to afford protection against the toxic action of the gonococcal endotoxin.[13] It will be of interest to determine whether this protection is based on goitrogenesis.

Another question arising from the data presented in this paper concerns the role and value of fever in resistance within the body to the toxemia of infections caused by Gram-negative bacteria. From our data it would seem that the heightened metabolism of the febrile state would enhance the toxicity of any antigen released during the course of an infection; and that low temperature or lowered metabolism would have an opposite and helpful effect. On the other hand, it is supposed that fever promotes within limits both antibody formation and phagocytic activity.[14] Whether in therapy one of these actions should be supported at the expense of the other, cannot be said in the absence of adequate quantitative information.

Summary. (1) An extensive list of pharmacological agents is presented, whose protective effects against the *Salmonella* endotoxin is reported as negative. The endotoxin of *S. typhimurium* was chosen for such tests because it is considered to be typical of the endotoxins generally found in Gram-negative bacteria. (2) Thiouracil and *p*-aminobenzoic acid have a limited protective effect against the lethal action of the endotoxin. Thiouracil protects against endotoxin-induced tumor hemorrhage. It is concluded that these effects, as well as the similar effects of the sulfonamides, is via a common mechanism, *viz.*, interference with thyroid activity. (3) Thiouracil has some protective effect against 1,2-dichloroethane, but is ineffective against colchicine. *p*-Aminobenzoic acid is likewise ineffective against colchicine. (4) Mice maintained at high environmental temperatures are more vulnerable to the endotoxin than those kept at lower temperatures. (5) The application of Selye's concept of "the general adaptation syndrome" to the action of certain poisons is briefly discussed.

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[12] Heppel, L. A., Neal, P. A., Perrin, T. L., Endicott, K. M., and Porterfield, V. T., *J. Pharm. and Exp. Therap.*, 1945, **84**, 53.

[13] Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 18.

[14] Perla, D., and Marmorston, J., *Natural Resistance and Clinical Medicine*, Little, Brown and Co., Boston, 1941, p. 1253.

15670 P

Growth Inhibition of Tubercle Bacilli by Fusarium, Sp.

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Growth of tubercle bacilli on media-containing egg or similar complex substances is inhibited by contamination of the medium with either fungi or bacteria. This inhibition is apparently not specific but results from the production of protein split product, deprivation of oxygen, etc.

On simpler mediums (*e.g.* Long's) the picture is very different. While some fungi are unable to grow on those mediums, others grow freely. As a rule they do not interfere with the growth of the tubercle bacillus and may even favor it.

A search was made for fungi capable of producing antibiotic agents. The following technic was used.

An alundun capsule (20 × 2 cm, dense) was introduced into a 250 cc Erlenmeyer flask containing 100 cc of Long's medium.

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* Died October, 1946.

The fluid inside the capsule (±5 cc) was inoculated with the fungus under investigation, while the outside fluid was inoculated with a recently isolated strain of virulent tubercle bacilli. While a rapid and easy exchange of metabolic product occurred through the alundun wall, the coarse fungus filaments did not as a rule penetrate it and invade the outer fluid. In the few cases in which this happened, the filtrate from the fungus growth was tested for the presence of growth inhibiting substances.

Of a large number of fungi, recently isolated from a variety of sources, only one, a Fusarium, sp., inhibited growth of the tubercle bacillus.

The inhibiting substance passes through fritted glass filters and Mandler filters but is partially inactivated by passage through Seitz filters. The crude filtrate is active up to a dilution of 1/20 and withstands heating to 100° for 15 minutes without loss of activity.

Experiments are now in progress to isolate the active principle and determine its activity *in vivo.*

C. D. Sherbakoff, Head Dept. of Plant Pathology, The University of Tennessee, Agricultural Experiment Station, has kindly identified the Fusarium as ***Fusarium scirpi*** Lamb. et Fautr. v. accuminatum (Ell. et Ev.) Wr. Wollenweber in his monograph on Die Fusarien, Z. f. Parasitenkunde, Berlin, 1931, places this Fusarium as No. 930 in a list of 1100.

15671 P

Urinary Excretion of Orally Administered Pteroylglutamic Acid.*

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Introduction. Few studies on the urinary excretion of pteroylglutamic acid have been reported.[1-4] No published information concerning the excretion of this vitamin after single large doses is available. Consequently, we are reporting results of such studies on 9 normal subjects and 9 hospitalized patients representing assays on 54 24-hour urine samples.

Methods. Since the primary purpose of the study was to determine the recovery of the free pteroylglutamic acid, no resort was made to the use of enzymes to liberate any conjugated vitamin.

One or more preliminary 24-hour urine samples were obtained in all but 2 instances. The test dose of synthetic pteroylglutamic acid was given orally (5.0, 5.1, 10 or 16 mg) and samples of urine were collected at varying intervals for the 24-hour period immediately following. The samples were preserved under benzene and aliquots were suitably diluted for determining the PGA microbiologically[5] with *Streptococcus faecalis*

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[1] Denko, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Friedman, T. E., and Youmans, J. B., *Arch. Biochem.*, 1946, **10**, 33.

[2] Denko, C. W., Grundy, W. E., Wheeler, N. C., Henderson, C. R., Berryman, G. H., Friedman, T. E., and Youmans, J. B., *Arch. Biochem.*, 1946, **11**, 109.

[3] Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

[4] Johnson, B. C., Hamilton, T. S., and Mitchell, H. H., *J. Biol. Chem.*, 1945, **159**, 425.

[5] Mitchell, H. K., and Snell, E. E., *The Univ. of Texas Publication*, 1941, No. 4137, 36.

TABLE I.

Preliminary Twenty-four-hour Urinary Excretion of Pteroylglutamic Acid.

Normal Subjects

Subject No.	Sex	24-hour excretion level pteroylglutamic acid, μg	Avg, μg
1	M	2.4; 2.9	2.65
2	M	2.4; 3.4	2.9
3	F	6.0; 2.8; 3.4	4.07
4	F	3.0; 1.5	2.25
5	M	2.0	2.0
6	F	2.7; 1.5	2.1
7	F	2.9	2.9
8	M	0.07	0.07
9	F	2.0	2.0
		Mean	2.34 μg
		Standard deviation	±1.08 μg

Hospital Patients

Subject No.	Sex	Diagnosis	24-hour excretion level pteroylglutamic acid, μg	Avg, μg
10	M	Pituitary deficiency	2.3; 5.5	3.9
11	M	Lymphatic leukemia	0.9; 1.1; 1.9; 1.4; 1.0; 2.3; 0.8	1.3
12	F	Hemorrhagic anemia	1.7	1.7
13	M	Myelogenous leukemia	1.0; 2.3; 1.8	1.7
14	M	Anemia	1.8	1.8
15	F	Lymphosarcoma	8.5; 4.7; 2.3; 2.6; 1.0; 1.3; 6.9; 11.9	4.9
16	M	Anemia	0.9; 0.7	0.8
17	M	,,		
18	M	Cardiac insufficiency		
			Mean	2.3 μg
			Standard deviation	±1.5 μg

(American Type Culture Collection No. 8043). Synthetic pteroylglutamic acid was used as the standard. Turbidimetric readings were made with a Coleman spectrophotometer.

Results. The assay values for the preliminary 24-hour periods are given in Table I. The mean value for 15 determinations on normal subjects was 2.34 γ ± 1.08 γ; that for the 24 studies on the hospitalized patients was 2.3 γ ± 1.5 γ. These values are in satisfactory agreement with results obtained by Denko *et al.*[1] and by Wright and Welch[3] on normal individuals.

Data on the rate of excretion of the test doses are given in Fig. 1. The data are presented in terms of the percentage of test dose excreted, since this appears to be independent of the size of the test dose used.

The curve is drawn through the cumulative average recoveries from the normal individuals for 4, 8 and 24 hours. The greater part of the excretion of the vitamin took place between the second and eighth hours following oral administration. The total 24-hour percentage recovery for the normal subjects averaged 28.5 (range 24.5-37.5%). With the exception of one patient who had received 5 mg of "Folvite"* 12 days previously, the total 24-hour recoveries for the 8 samples from hospital patients fell below 11% (range for all patients 1.75-26.9%). The diagnoses and the preliminary urinary pteroylglutamic acid excretion levels are presented in Table I. The surprisingly small percentage recoveries of the vitamin in these patients may indicate either a low degree of saturation, increased destruction, increased requirement, or the conjugation of pteroylglutamic acid before excretion. The data at hand do not permit a choice among these possibilities.

Summary. Fifty-four urine samples for 24-hour periods have been assayed microbiologically for free pteroylglutamic acid. For normal subjects the average 24-hour excretion was 2.34 γ. Following an oral dose of pteroylglutamic acid the mean percentage recovery was 28.5%. Although the hospitalized patients studied had a similar excretion level (average 2.3 γ for 24 hours) prior to dosage with pteroylglutamic acid, the per-

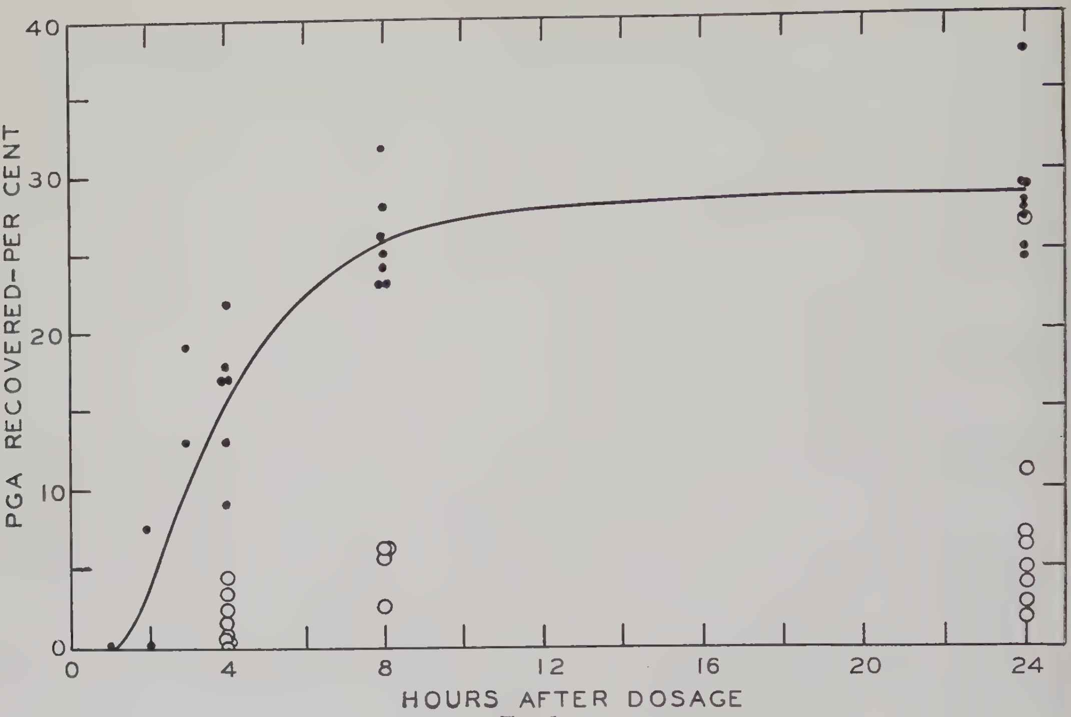

FIG. 1.
The percentage urinary recovery of pteroylglutamic acid after single oral doses of 5.0, 5.1, 10 or 16 mg. ●—Normal subjects; ○—hospital patients.

centage returns from single oral doses were much lower than in the normal subjects, a finding which is possibly indicative of a low degree of saturation.

15672 P

Effect of Folic Acid upon Primitive Erythrocytes *In vitro*.*

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Since the antianemic properties of pteroylglutamic acid (synthetic folic acid) were reported[1] there have been many studies to determine how this substance acts upon the bone marrow to stimulate the production of cells and to ascertain the relationship of pteroylglutamic acid to the antipernicious anemia principle. Efforts to demonstrate significant amounts of pteroylglutamic acid in highly potent antipernicious anemia liver fractions have failed.[2,3] Attempts made to liberate free pteroylglutamic acid from ground muscle treated with normal gastric juice according to the technic of Castle and Town-

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* Aided by a grant from The Armour Laboratories, Armour and Company, Chicago, Ill.

† With the technical assistance of Miss Betty Paulsen, Mrs. Francena Galbraith, and Miss Constance Brownell.

[1] Spies, Tom D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *Southern Med. J.*, 1945, **38**, 707.

[2] Clark, Guy W., *Am. J. Med. Sci.*, 1945, **209**, 520.

[3] Stokstad, E. L. R., and Jukes, T. H., PROC. SOC EXP. BIOL. AND MED., 1946, **62**, 112.

TABLE I.

Substrate added to Tyrode solution*	Final conc., μg per ml	No. of reticulocytes per 1000 nucleated cells	
		Avg†	Range
None	—	7 (20)	3-8
Pteroylglutamic acid (folic acid)	4.9	8 (2)	8-8
	.05	8 (4)	6-9
	.0005	8 (2)	5-10
	.00005	7 (2)	7-7
Hexaglutamyl-pteroylglutamic acid (vitamin B_c conjugate)	1.0	8 (2)	7-9
	.1	7 (2)	7-7
	.01	6 (4)	5-8
Rat serum	—	13 (20)	9-34
Normal human serum	—	15 (4)	9-24
Human pernicious anemia serum‡	—	7 (1)	—
Human pernicious anemia serum‡ and liver extract	.005	13 (1)	—
Human pernicious anemia serum‡ and pteroylglutamic acid	.0025	8 (1)	—
Liver extract§	.1	11 (6)	6-18
	.01	11 (16)	9-18
	.001	13 (1)	—
	.0001	10 (2)	7-14
	.00001	6 (2)	6-6

* All substrates were dissolved in glucose-free Tyrode solution. In the cases where serum was added, 1 volume of serum was added to 3 volumes of glucose-free Tyrode solution.

† Numbers in parentheses indicate number of different experiments.

‡ Taken from case of pernicious anemia in relapse.

§ Liver extract powder provided by The Armour Laboratories. This extract (Lot No. 11285A) was used in the preparation of a 15 U.S.P. unit liver extract. 17 mg was equivalent to 1 U.S.P. unit.

send[4] in this and other laboratories likewise have failed. It would appear likely, therefore, that either the antipernicious anemia principle produced by the interaction of Castle's "extrinsic" and "intrinsic" factors, and that stored in the liver, are free of pteroylglutamic acid; or that pteroylglutamic acid is a component of the antipernicious anemia factor which ordinary methods of hydrolysis and assay fail to liberate and detect. The report of Welch *et al.*[5] that gastric juice did not liberate free pteroylglutamic acid from the heptaglutamyl (vitamin B_c Conjugate) form would support the latter view. From this report, there also appears to be some difference between the pernicious anemia patient and the normal individual with respect to the ability to break down the conjugated form to free pteroylglutamic acid. This finding agrees with the report of Bethel *et al.*[6] that patients having pernicious anemia fail to excrete increased amounts of pteroylglutamic acid in the urine when the heptaglutamyl conjugate form is fed. On the other hand, normal individuals are able to convert the conjugate form into pteroylglutamic acid and excrete it. In both instances pteroylglutamic acid when administered orally causes a marked increase in the urinary excretion of free pteroylglutamic acid.

In spite of the similarity between the clinical response obtained from the administration of antipernicious anemia liver fractions and pteroylglutamic acid in the treatment of the various blood dyscrasias it is becoming apparent that the actual stage at which these 2 substances stimulate erythropoiesis may not be the same. In addition, it is known that on a weight for weight basis, liver extracts are more potent than pteroylglutamic acid or its heptaglutamyl form.

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[4] Castle, W. B., and Townsend, W. C., *Am. J. Med. Sci.*, 1929, **178**, 764.

[5] Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V., *J. Biol. Chem.*, 1946, **164**, 787.

[6] Bethell, F. H., Swendseid, M. E., Bird, D. D., Meyers, M. C., Andrews, G. A., and Brown, R. A., *Univ. Hosp. Bull., Univ. of Michigan*, 1946, **12**, 42.

The work reported here is the result of a series of experiments that has been conducted in this laboratory during the past year comprising a study of the effect of pteroylglutamic acid,‡ its heptaglutamyl conjugate form,§ and liver fractions highly potent with respect to their antipernicious anemia content upon red cell maturation *in vitro* in an attempt to secure information concerning the point at which these substances act upon red cell production. After removing the immature cells from other marrow constituents by means of centrifugation the resulting preparation permits a study of the maturation process independent of the modifying factors of the gastro-intestinal tract, the liver, and the bone marrow matrix which are found in the intact animal and hence it is possible to study this one phase of red cell production separately.

Table I summarizes some of the results which were obtained by using bone marrow cell survival technic based upon the method of Osgood and Brownlee.[7,‖] Rat bone marrow cells were incubated 3-5 hours at 37°C while suspended in a glucose-free Tyrode solution. At the end of the incubation period, reticulocyte counts were made using the wet preparation technic and staining with brilliant cresyl blue. Only "mature" reticulocytes were counted. Lang-Levy micropipettes[8] were used in adding the various constituents to the media. A standard liver extract of known concentration was included in each day's experiment.

It will be noted that neither pteroylglutamic acid nor its heptaglutamyl conjugate, appeared to bring about maturation of red cells from their primitive precursors to the mature reticulocyte stage. On the other hand, a liver preparation produced maturation quantitatively similar to that change observed when normal serum is added to the preparation. It should be noted that in a single instance serum from a patient with pernicious anemia in relapse did not show the presence of this maturation factor. It is possible that this experiment may indicate that the stimulant for erythropoiesis found in the blood is similar to that found in liver extract and not similar to pteroylglutamic acid.

The difference between the effect of liver extracts and of pteroylglutamic acid upon immature bone marrow cells has been noted in the presence of glucose, casein hydrolysate, or glucose and casein hydrolysate in the same medium so that it appears not to be a function of a metabolite or a combination of amino acids present. It has been observed that ferrous iron added to any of the above substrates appears to diminish maturation.

Summary. The above evidence indicates that pteroylglutamic acid, or its conjugated form, does not act directly in an unaltered state upon primitive red cells to mature them, whereas a highly potent antipernicious anemia liver preparation appears to cause a maturation comparable to that observed in the presence of serum. A single sample of serum from a patient having pernicious anemia in relapse appeared to lack the maturation factor found in normal human and rat serum. Pteroylglutamic acid failed to increase the maturation of bone marrow cells suspended in this serum.

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‡ Courtesy of Lederle Laboratories, Pearl River, N.Y.

§ Courtesy of Dr. J. J. Pfiffner, Parke, Davis and Co., Detroit, Mich.

[7] Osgood, E. E., and Brownlee, Inez E., *J. A. M. A.*, 1936, **107**, 123.

‖ Details of this method are to be published.

[8] Levy, M., *C. R. trav. Lab. Carlsberg, Serie chim.*, 1936, **21**, 101.

15673 P

Bacterial Variations in Salicin Medium.

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Most investigators have concerned themselves with the variation in morphological and colonial characteristics, especially in regard to the smooth (S) and rough (R) types. Neisser,[1] in his first work on variation, noted that a nonlactose-fermenting, colon-like organism gave daughter colonies which fermented lactose. Burri[2] observed this same phenomenon in regard to sucrose fermentation, when he studied an organism isolated from fermenting grass.

Many investigators have found no characteristic biochemical difference among their variants when the original culture was grown in media containing carbohydrates or higher alcohols except lactose.

Variation occurs spontaneously under ordinary conditions of cultivation, and also with environmental stimuli such as the presence of sugars, dyes, or changes in temperature. The presence of salicin in the medium as the environmental stimulus was chosen in this present investigation. The chief object was to study the characteristics of the variants which were produced when separate individual colonies from the mother culture were successively transplanted in salicin broth for 15 times.

A group of 36 salicin-negative organisms were selected, 31 of these belonging to the *Escherichia* genus and 5 to the *Aerobacter* genus. Eosin-methylene blue plates were streaked from these cultures, and 10 colonies which showed no gas production in salicin medium were transferred every third day to fresh tubes of salicin medium for at least 15 times. The percentages of gas produced were recorded after 7 days incubation at 37°C. As an example, a record of the acquired ability of culture *E. anindolica* G. to ferment salicin is shown in Table I.

Of the 36 organisms used, 22 (220 sub-cultures) remained negative to salicin throughout the 15 transplants in salicin broth. Of the other 14 organisms (140 sub-cultures), there were from 1 to 10 of the sub-cultures of each organism which acquired the ability to ferment salicin after from 1 to 12 transplants. Seventy-six of these 140 sub-cultures fermented salicin and 64 remained negative. Ten of the 64 salicin-negative sub-cultures were streaked on eosine methylene blue agar plates. Ten colonies were selected from each plate and placed in salicin medium. Positive variants were obtained from 7 of the salicin-negative daughter colonies. In no instance, however, did more than one tube out of 10 representing a given culture produce more than 10% gas. In many instances, it was discovered that the salicin-negative and salicin-positive variants from the same culture gave different forms of colonies on agar plates.

A representative number of the salicin-positive variants were placed on agar slants for permanent cultures. All of these cultures were transferred to salicin broth after being stored for 3 years at about 4°C. In every instance, where the organisms were still alive, the reaction to salicin was still positive. Streaks of 20 of the cultures were made on eosin-methylene blue agar. Ten of the individual colonies from each culture were grown in salicin medium for 7 days. The majority of the sub-cultures produced gas in salicin medium.

The quantitative study of the progressive acid fermentation was conducted on the original culture of *Aerobacter cloacae* and on one of its variants which developed the power to ferment salicin. These 2 organisms were grown in salicin medium, and at frequent intervals during 180 hours, the pH values of the medium were determined. Similar determinations were made for a culture of *Escherichia formica* and one of its salicin-

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[1] Neisser, M., *Centralbl. f. Bakt.*, Abt. I, Ref., 1906, **38**, 98.

[2] Burri, R., *Centralbl. f. Bakt.*, Abt. II, 1910, **28**, 321.

TABLE I.
Production of Gas in Salicin Medium of Variants of *E. anindolica* G.

No. of transplant	Daughter colonies									
	1	2	3	4	5	6	7	8	9	10
1	0*	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	5	10	0	20	10	0	0	0	0	5
7	10	10	5	25	15	0	0	0	0	15
8	10	10	5	25	30	0	0	0	20	10
9	20	20	15	25	25	0	20	0	25	20
10	20	25	25	20	15	5	15	0	20	15
11	20	25	35	20	15	20	25	0	20	30
12	30	20	30	20	15	20	20	20	20	35
13	35	35	30	25	15	20	20	30	20	40
14	35	35	20	25	15	30	25	35	20	40
15	35	35	30	30	25	30	30	35	35	40

* Figures are in percentages of gas produced in 7 days.

positive variants.

It was discovered that the salicin-positive variants developed a greater capacity to form acid from salicin. The original cultures (salicin-negative) showed an acid production for about 10 hours to a minimum pH of about 6.7, after which there was an abrupt rise in pH to over 8.5 during 180 hours. The salicin-positive variants from these cultures reduced the pH value of the medium to a value around 5 in 10 to 30 hours. The acid production was so great that the organisms were killed in most instances.

The results of this investigation indicate that, the fermentation of a given substance, such as salicin, is not necessarily a constant characteristic. Because of the acquired ability to ferment this glucoside, a number of organisms changed from one species to another according to the Bergey classification. Five cultures change their classification from *E. anindolica* to *E. communior*, 3 from *E. formica* to *E. coli*, and 3 from *E. gruenthali* to *E. paragruenthali*.

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High Molecular Weight Polyglycols as Reagents in the Gravimetric Determination of Inorganic Phosphate.

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Despite the availability of the simpler, direct colorimetric methods for the determination of small amounts of inorganic phosphate in biological materials, gravimetric methods, by reason of their greater precision and accuracy, have continued to find some application. Two precipitates that have been employed for quantities of phosphate too small for the magnesium ammonium phosphate method are the ammonium and strychnine salts of phosphomolybdic acid. The strychnine phosphomolybdate appears to have gained wider preference; its advantages over the ammonium salts have been summarized by Peters and Van Slyke[1] as follows: (1) the weight of the precipitate is greater, for per 1 mg of P the ammonium phosphomolybdate weighs 69 mg and the strychnine phosphomolybdate 89.3 mg; (2) the strychnine precipitate is more insoluble and forms much more rapidly; (3) the strychnine precipitate readily coagulates into large particles which can be more easily handled than the ammonium precipitate and have less tendency to adhere to the walls of the precipitating vessel; and (4) the strychnine precipitate can be dried to constant weight in an ordinary drying oven at 100° to 110°.

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[1] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Vol. II, Methods, pp. 849, 873, Baltimore, The Williams and Wilkins Co., 1932.

With the discovery that the solid polyethylene glycols form highly insoluble complexes with phosphomolybdic acid in the presence of barium,[2] the possibility suggested itself that these compounds might be adapted to a gravimetric determination of phosphate even more advantageously than strychnine. Subsequent experimentation has justified this expectation, and the present report describes the results obtained with the substitution of a high molecular weight polyglycol for strychnine in the Embden-Fetter method for the determination of inorganic phosphate in blood and urine. The barium-polyglycol-phosphomolybdate precipitate is the equal of strychnine phosphomolybdate on each of the last 3 counts mentioned above, and is superior in that it weighs 97.1 mg per mg of P. Its sole disadvantage is that its formation requires the presence of barium and, conversely, the absence of sulfate. This interference, however, may readily be eliminated by a precipitation preceding the filtration that accomplishes deproteinization of the sample. Minor considerations in favor of the use of the polyglycols are their low cost and availability since they are synthetic chemicals produced in tonnage capacity.

Reagents. All reagents employed, with the exception noted, are those specified in the Embden-Fetter method.[1] The polyglycol used is a commercial product sold under the trademark* name of "Carbowax" compound 6000, the numeral indicating a mixture of polymers with a mean molecular weight of 6000. As purchased, this material contains a small quantity of phosphate as an impurity, which may be removed by adsorption on basic ferric acetate in the following manner: Twenty g of the polyglycol are dissolved in *ca.* 160 ml of water and the solution is made faintly acid to methyl red by the addition of a few drops of dilute acetic acid. Then there are added in succession 4 ml of a 10% solution of ferric chloride in 0.2 N hydrochloric acid and 8 ml of a 5% solution of ammonium acetate. The mixture is heated with continual agitation until boiling just begins, at which point it is cooled momentarily and a few drops of dilute ammonium hydroxide are added. The voluminous precipitate which forms is immediately filtered. The filtrate, which is the polyglycol solution to be employed in subsequent work, should be water-white or of only a very faint greenish color, and should give no turbidity upon mixing with the molybdate solution. The precipitating reagent is prepared within an hour of use by mixing one volume of the polyglycol solution with three volumes of the molybdate reagent, in the same manner as the strychnine molybdate is prepared.

No sulfate was encountered in either of the two samples of "Carbowax" compound 6000 used in this work; however, had this anion been present, it would obviously have been necessary to have removed it prior to employment of the reagent. This removal could probably be best effected by acidifying the original polyglycol solution, adding barium chloride, and filtering with suction through fine asbestos. The reaction of the filtrate would then be adjusted with dilute ammonium hydroxide, and the removal of phosphate carried out from that point.

Procedures. (A) For Blood. Five ml of blood, plasma, or serum are measured into a 50 ml flask containing 25 ml of 10% trichloracetic acid. Following a thorough shaking of the coagulum, 5 ml of a 10% barium chloride solution are added, and the flask is filled to the mark with water. After about 15 minutes of standing, the contents are filtered through Whatman No. 42 or comparable paper. Twenty-five ml of the filtrate are diluted to 60 ml, and 20 ml of the polyglycol-molybdate solution are added. The mixture is allowed to stand at room temperature for one hour

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[2] Shaffer, C. B., and Critchfield, F. H., *Ind. Eng. Chem., Anal. Ed.*, 1947, in press.

* Carbide and Carbon Chemicals Corporation.

TABLE I.

Comparison of Phosphate Determinations on Random Samples of Plasma and Urine Performed by the Reference Method and Duplicated by the Proposed Method.

	Phosphate P per 100 ml			
	Plasma		Urine	
Sample No.	Strychnine mg	Polyglycol mg	Strychnine mg	Polyglycol mg
1	5.15	5.36	79.14	80.55
2	3.72	3.72	115.12	117.52
3	4.37	4.15	4.07	4.02
4	3.52	3.44	69.41	68.91
5	4.44	4.64	27.42	28.02
6	3.52	3.52	56.49	57.06
7	3.56	3.52	169.83	172.21
8	2.82	3.08	32.08	32.14
9	3.78	3.60	70.71	71.07
10	2.90	2.84	109.67	109.18
11	3.38	3.52	29.85	30.38
12	2.90	3.04	77.26	79.00
13	4.68	4.92	86.02	85.49
Mean	3.75	3.80	71.31	71.96

with occasional shaking to complete the precipitation. The precipitate is then filtered in a small Gooch crucible previously dried at 110°, cooled in a desiccator, and tared. It is important to use only gentle suction during filtration. Particles of the precipitate which adhere to the container are rinsed into the crucible with 25 ml of ice-cold, diluted (1:5) molybdate solution. The precipitate is then washed with ice-cold water until the washings are no longer acid. During the washing, as soon as one portion of fluid has been drawn through the crucible another portion is added or else the suction is stopped, to prevent the precipitate from becoming divided by cracks through which subsequent portions of wash water can run with little contact with the precipitate. The crucible is dried for an hour at 100°-110°, cooled in a desiccator, and weighed.

(B) For Urine. Five ml of urine, well mixed to suspend any insoluble phosphates, are measured into a 50 ml volumetric flask together with 10 ml of water and 25 ml of 10% trichloracetic acid. Five ml of a 10% solution of barium chloride are then added dropwise, and the flask is allowed to stand undisturbed for one-half hour. At the end of this time the contents are shaken and made up to the mark with water. The barium sulfate precipitate, together with any coagulum in the flask, is separated by filtration, or by centrifugation if necessary, and an aliquot of the clear filtrate (usually 10 to 20 ml depending upon phosphate content) is diluted to 60 ml with water, 5 ml more of the barium chloride solution are added, and the rest of the determination is carried out as described above for blood.

(C) Determination of gravimetric factor. Just as in the case of the strychnine precipitate, each analyst should determine for himself the factor indicating the mg of P per mg of barium-polyglycol-phosphomolybdate precipitate. It has been found that different preparations of strychnine may give different weights of precipitate per mg of P, and it may be assumed that less homogeneous polyglycols will show some limited variation in this respect, although samples from 2 different production batches tested in this work gave identical results. For determination of this factor, a standard phosphate solution containing 0.4395 g/l of thoroughly dried reagent grade KH_2PO_4 was prepared. Aliquots containing 0.5 to 1.5 mg of P (5 ml to 15 ml of solution) were measured from a certified buret, diluted to 60 ml with water, 5 ml of barium chloride solution were added, and the phosphate was precipitated and weighed in the manner described. From a series of 15 trials the gravimetric factor was calculated

as 0.0103.

Results. Table I lists the results of inorganic phosphate determinations of 13 different samples of dog plasma and 13 different samples of human urine carried out by the proposed method, as compared with duplicate determinations performed simultaneously on the same specimens by the Embden-Fetter strychnine-phosphomolybdate method. Statistical treatment of these values by the application of the t distribution indicated no significant difference between the results obtained.

In the analysis of whole blood, correlation of the results obtained by the two methods on a given sample is good provided that filtration of the precipitate is performed promptly at the conclusion of the one-hour period of standing recommended. However, when this time of standing is prolonged, hydrolysis of organic phosphate appears to take place more extensively in the strychnine-precipitated sample and to make the results from this procedure high as compared with those from the other.

Summary. A method is described for the precipitation of phosphate phosphorus as a barium-polyglycol-phosphomolybdate complex which may be filtered and dried to constant weight at 100°-110°. Evidence is presented to show the applicability of this reaction to phosphate determinations on biological materials, and a comparison is made of this procedure with the strychnine phosphomolybdate (Embden-Fetter) method.

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Effects of Vital Dyes on Early Development of the Amphibian Embryo.

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A vital dye may be used to stain different cells and regions of eggs and embryos. To be successfully employed in this fashion, the dye should be "non-toxic and harmless so as not to destroy or even impair the vital activities and embryonic performance of its carrier" (Weiss[1]). However, it has been shown by Child and his co-workers,[2] in the work on the occurrence of physiological gradients, that so-called "vital" dyes, especially the basic ones, can be quite toxic. Determination of the conditions under which vital staining of eggs and embryos can be successfully accomplished is of interest. The general effects of two basic dyes, Nile Blue Sulfate and Neutral Red, were studied by Detwiler[3] in Amblystoma embryos. Gersch[4] and Gersh and Ries[5] in their work on sea-urchin eggs, found that different concentrations of the acid dye, Trypan Blue, caused severe developmental anomalies. Inhibition of mitosis was shown to occur in the larvæ of *Triton taeniatus* after a one- to two-hour exposure to certain concentrations of Neutral Red (Luther[6]). However, Uschin[7] who studied the same problem in *R. temporaria* tadpoles found no dye effects on mitosis.

In view of the frequency with which the

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* Present address, Department of Biochemistry and Physiology, Washington University School of Dentistry, St. Louis, Mo. Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Weiss, P., *Principles of Development*, 1939, Henry Holt and Co., New York.

[2] Child, C. M., *Patterns and Problems of Development*, 1941, The University of Chicago Press.

[3] Detwiler, S. R., *Anat. Rec.*, 1917, **13**, 493.

[4] Gersch, M., *Arch. Entw. Mech.*, 1937, **136**, 210.

[5] Gersch, M., and Ries, E., *Arch. Entw. Mech.*, 1937, **137**, 169.

[6] Luther, W., *Klin. Woch.*, 1939, **18**, 682.

[7] Uschin, N. P., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 472.

eggs of the leopard frog, *Rana pipiens,* are used in experimental embryology studies, an investigation of their response to exposure to vital dyes under different conditions seemed of practical importance.

Materials and Methods. The dyes used were Nile Blue Sulfate, Neutral Red and Bismark Brown. Fresh stock solutions containing 100 mg of commercial dye[†] in 1000 cc of solvent (spring water[‡]) were prepared before each experiment and diluted as required. Eggs of *R. pipiens* (Vermont stock) were obtained in the laboratory by the method of induced ovulation and fertilization described by Rugh[8] and were allowed to develop in finger bowls containing 200 cc of solution; between 20 and 30 eggs per bowl. A total of 2050 eggs were used. Throughout the course of the experiments, the eggs were studied for both character and rate of development. This was recorded in terms of stages of normal development of *R. pipiens* as described by Shumway.[9]

Observations. I. Concentration. Eggs were placed in dye solutions of different concentrations ranging from 1:10,000 to 1:1,000,000 while in the 2-cell stage (st. 3) and were allowed to develop therein up to the complete operculum stage (st. 25). For each dye it was possible to determine a "critical" concentration below which development was completely normal and above which abnormalities and lethal effects began to appear. This critical point varied with each dye, being lowest with Nile Blue Sulfate (1:750,000), considerably higher with Neutral Red (1:250,000) and just slightly higher with Bismark Brown (1:100,000).

Developmental abnormalities produced by exposure to concentrations above the critical levels were of the same nature for each of the dyes. A direct correlation between concentration and time of appearance of the abnormalities seemed to exist; the stronger the concentration the sooner development became abnormal, up to a certain point. However, even with the strongest concentrations used, 1:10,000 (below this point the eggs became so heavily stained that it was impossible to see the cleavage furrows) cleavage was always normal in both character and rate.

The gastrulation stages, beginning with the appearance of the dorsal lip of the blastopore (st. 10) were the first stages in which an effect on development could be noted. In many instances development stopped entirely immediately after the formation of the dorsal lip; in others, development continued but in an abnormal fashion. The effect seemed to be one which involved an interference with the growth of the cells of the animal hemisphere over the large yolk-laden cells of the vegetal hemisphere. Consequently masses of yolk material remained exposed.

All embryos with exposed yolk material which survived beyond gastrulation showed imperfect neurulation. This consisted of an interference with either the formation or the closure of the neural folds. In most cases neural folds could form in the anterior region, and therefore the state of development found in that region was used as a basis for the classification of the embryos into stages. All degrees of abnormalities were found from the cases in which the entire dorsal region of the embryo was occupied by yolk material to those in which only a small yolk plug blocked the closure of the neural folds in the posterior region.

II. Timed exposure to vital dyes. In order to determine, for each concentration, when

TABLE I.

For each concentration is given the length of exposure in hours which did not interfere with normal development. Eggs exposed at stage 3 and maintained at 19 ± 1.5°C. (220 hours represent the time needed to reach st. 25, the end point of these experiments).

Concentration × 1000	Nile blue sulfate	Neutral red	Bismark brown
1:25	—	<3	90
1:50	<3	6	150
1:75	—	12	—
1:100	12	72	175
1:250	24	220	220
1:500	110	220	220
1:750	175	220	220
1:1,000	220	220	220

[†] Obtained from the National Aniline and Chemical Company, N.Y.

[‡] Purchased from the Great Bear Spring Company, N.Y.

[8] Rugh, R., *Experimental Embryology, A Manual of Techniques and Procedures,* 1942, New York University Bookstore, New York, N.Y.

[9] Shumway, W., *Anat. Rec.,* 1940, **78**, 139.

TABLE II.
Fifteen Hours Exposure to Nile Blue Sulfate.

Hr after fertilization when immersed	Stage No. and description at time of immersion Control and experimental	Description at time of removal. Control and exper.	% showing any abnormality		% surviving through St. 25	
			1:150,000	1:100,000	1:150,000	1:100,000
1	2—grey crescent	mid-cleavage	0	29	97	71
6	6—16 cells	” ”	0	25	98	81
22	9—late cleavage	mid-gastrula	48	84	62	33
26½	10—dorsal lip	late ”	17	79	78	28
39	11—mid-gastrula	early neural plate	1	80	98	75
45½	12—late gastrula	” ” folds	0	76	98	72
63	14—neural folds	neural tube	0	3	100	97

the dye accumulating within the cells reached a concentration incompatible with normal development, eggs immersed at the 2-cell stage, were allowed to remain for graded lengths of time, 3, 6, 12, 18, 24, 36, 48, 67 hours, and then were transferred to fresh spring water for further growth and development. A summary of the maximum exposure to different concentrations which did not interfere with normal development is given in Table I.

A result which might be due to a delayed effect of exposure to the dyes was observed in this series. Eggs exposed for short periods of time to the stronger concentrations developed normally while in the dye solutions and also for some time after removal to fresh medium, but then suddenly became abnormal at the time of gastrulation. For example, eggs which were exposed to 1:50,000 Nile Blue Sulfate from the 2-cell stage to a many-cell stage (a 6-hour exposure) developed normally through this period and for approximately 18 additional hours in fresh spring water. At the end of these 18 hours the eggs reached the dorsal lip stage and the abnormalities already described began to manifest themselves.

III. Effect of stage of development on results of exposure to dyes. Since the first observations indicated a great sensitivity of gastrular stages, a separate study was made of the influence of developmental stage on the results. Eggs were exposed to different concentrations of Nile Blue Sulfate for 15 hours while at different stages of development. In Table II are given the number of hours after fertilization and a description of the development at the time of exposure and removal from the dye solutions.

Of all the concentrations used, 1:100,000 and 1:150,000 were found to be critical for a demonstration of the phase susceptibility (Table II, columns 4, 5, 6, 7). Exposure starting at least one hour before the first cleavage did not delay the onset of division or affect the rate or character of the following divisions. Any departures from the normal occurred at gastrulation, long after removal from the dye. The results were similar for eggs exposed at 6 hours after fertilization (st. 6). Starting at st. 9 an increase in the sensitivity of the eggs was noted as indicated by a rise in the percentage of eggs showing abnormalities as compared with the st. 2 and st. 6 eggs. In a concentration of 1:150,000, the percentage of cases of abnormality decreased in stages 11 and 12. In the higher concentrations of 1:100,000 where it is quite likely that the rate of penetration of dye per unit time was higher, the percentage of abnormality remained large. Nevertheless, the decrease in susceptibility was demonstrable, since the abnormalities produced were less severe and a higher percentage of survival resulted. Of the eggs exposed at st. 11 and st. 12, 75 and 72% respectively were able to survive to st. 25 (the endpoint of the experiment) while of the eggs exposed at st. 9 and st. 10 to the same concentration for the same length of time only 33 and 28% respectively reached the same point. No effect on development was noted when exposure was made at st. 14.

Thus it would seem that in the development of eggs through the neurula stage, late cleavage and dorsal lip stage (st. 9 and st. 10), were the most sensitive to the presence of dye since exposure at these times produced the largest number of abnormalities and the poorest survival. The question is raised as to whether the high sensitivity seen at st. 9 might not have been due to the fact that in the 15-hour period of exposure, the eggs developed through st. 10 while still in the dye.

IV. Effect of hydrogen ion concentration. The pH of the dye solutions used was found to vary from pH 6.8-6.9 at the time of preparation and to change within a period of 24 hours to pH 7.3-7.4 (probably due to the alkalinity of the glassware used). Therefore, dye solutions buffered to different pHs from pH 4.5 to pH 8.5 were prepared and the staining results compared with those of the unbuffered solutions. It was found that even at high concentrations development proceeded normally through st. 25 in the most acid solutions and that the larvae were only very faintly stained even after long periods. At pH 6.3 to 8.5 the effects were the same as have been described for the unbuffered solutions and also the larvae were all very heavily stained. This agrees substantially with the observations of MacArthur[10] on the staining of *Planaria dorotocephala* with basic dyes and also with the work of Chambers, Irwin and others[11] on the effects of pH on the penetration of dyes into unicellular forms. The phenomenon can be explained either on the basis of the dissociation of the dyes or on the basis of the effect of pH on the particle size (Gordon & Chambers).[12]

Discussion. It has been shown that unless the concentration used, the length of exposure, the stage of development of the eggs, and the hydrogen ion concentration are carefully considered, exposure of the eggs of *R. pipiens* to solutions of vital dyes, Nile Blue Sulfate, Neutral Red and Bismark Brown, may result in abnormal development. This is especially true when the early cleavage stages are used because although cleavage itself remains unaffected the stages which follow are severely damaged.

Nile Blue Sulfate produces good staining but must be used in very low concentrations. Neutral Red is considerably less toxic and Bismark Brown is even less so. As far as could be determined here, there is no difference in the affinity of the 3 dyes for the eggs used. All 3 can readily be seen in fixed sections and also in the living state even though the eggs of *R. pipiens* contain a large amount of dark pigment which has a tendency to mask the color somewhat. These facts would seem to indicate the advisability of using Neutral Red and Bismark Brown in experiments which involve the staining of entire eggs and embryos. The best staining results are obtained by long exposures to weak concentrations rather than by short exposures to strong concentrations. Better results are also produced if the eggs are exposed to the dyes without removal of the jelly capsule. This was also noted by Detwiler[3] for the eggs of Amblystoma. The color produced is uniform throughout the eggs, and the ectoderm is perfectly normal.

Summary. 1. Eggs of *Rana pipiens* were exposed to solutions of the vital dyes Nile Blue Sulfate, Neutral Red and Bismark Brown under different conditions. Concentrations less than the following allowed normal development of eggs and embryos: Nile Blue Sulfate 1:750,000; Neutral Red 1:250,000; Bismark Brown 1:100,000.

2. Developmental abnormalities appeared with use of stronger concentrations than listed above.

3. Early cleavage was unaffected by otherwise toxic concentrations even to 1:10,000. Gastrulation proved to be the critical point in development when toxic effects of the dyes began to produce abnormalities.

4. Short exposure to toxic concentrations, even during unaffected cleavage, resulted in abnormalities appearing at subsequent stages of development.

5. Developmental stages 9 and 10 were more susceptible to toxic effects than were stages 2, 6, 11, 12, 14 using Nile Blue Sulfate of 1:100,000 and 1:150,000 concentrations.

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[10] MacArthur, J. W., *Am. J. Physiol.*, 1921, **57**, 350.

[11] See review of Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, **1943**, Cambridge University Press.

[12] Gordon, H. K., and Chambers, R., *J. Cell. Comp. Physiol.*, 1941, **17**, 97.

6. The pH of the medium was shown to influence the toxicity effects.

7. When Nile Blue Sulfate, Neutral Red and Bismark Brown are used as vital dyes, the factors of concentration, length of exposure and stage of development must be considered to avoid induced abnormalities in development, most of which begin at the time of gastrulation.

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A Method for Producing Experimental Venous Thrombosis.*

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In our attempts to study the genesis and functional pathology of intravascular thrombosis, and to determine the *in vivo* action of heparin on preformed clot, it was found necessary to devise a method of experimental clot formation in animals which produced consistent, predictable results. This report deals briefly with such a method successfully employed in rabbits.

Experimental venous thrombosis has hitherto been accomplished by chemical and mechanical means.[1–4] The introduction of an extraneous factor in chemically-induced thrombosis unnecessarily complicates the proper evaluation of the effect of heparin. Accordingly, mechanical methods of inducing thrombosis are preferable. Vein crushing over an intraluminal silk thread,[5] although reported to be 80% successful, did not give us consistently good results. Similarly, we found stretching of the vein to induce intimal damage[6] successful in only 20% of instances.

The elaboration of a thrombus depends on (a) stagnation of blood, (b) injury to the intima, (c) local release of considerable amounts of thrombokinase. A method utilizing these 3 factors is herewith described. Three kilogram adult rabbits are anesthetized with ether and a midline cervical incision is made. The jugular veins on either side are exposed. Both veins are treated alike. A 3-cm segment of vein is dissected free and the most proximal portion securely tied with a silk ligature. A flat, narrow strip of metal, such as a ribbon retractor, is placed under the vein distal to the ligature and acts as an anvil. The vein is then given 15 to 30 sharp taps with the handle of a Mayo scissors. Brisk bleeding will occur which is readily controlled by gauze pressure. Care is taken not to fracture the vein completely across. When bleeding has ceased, usually in about 2 minutes, a palpable and visible thrombus appears. If clotting does not occur, the procedure is again repeated. Clotting invariably is present after the second series of strokes. All animals are reexamined after 48 hours to reaffirm the presence of clots. Examination and study of these clots, in the gross and in microscopic sec-

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* Aided by a grant from the William R. Warner & Co., Inc., New York.

[1] Zahn, F. W., *Virchow's Arch. f. path. Anat.*, 1875, **62**, 81; *ibid.*, 1884, **96**, 1.

[2] Eberth, J. C., and Schimmelbusch, C., *Virchow's Arch. f. path. Anat.*, 1886, **103**, 39; *ibid.*, 1886, **105**, 456.

[3] Welch, W. H., *Papers and Addresses*, Vol. 1, p. 110, Baltimore, 1920, Johns Hopkins Press.

[4] Zurhelle, E., *Zeigler's Beitr. z. path. Anat. u. z. allg. Path.*, 1910, **47**, 539.

[5] Murray, D. W. G., Jaques, L. B., Perrett, T. S., and Best, C. H., *Surgery*, 1937, **2**, 163.

[6] Rabinovitch, J., and Pines, B., *Surgery*, 1943, **14**, 669.

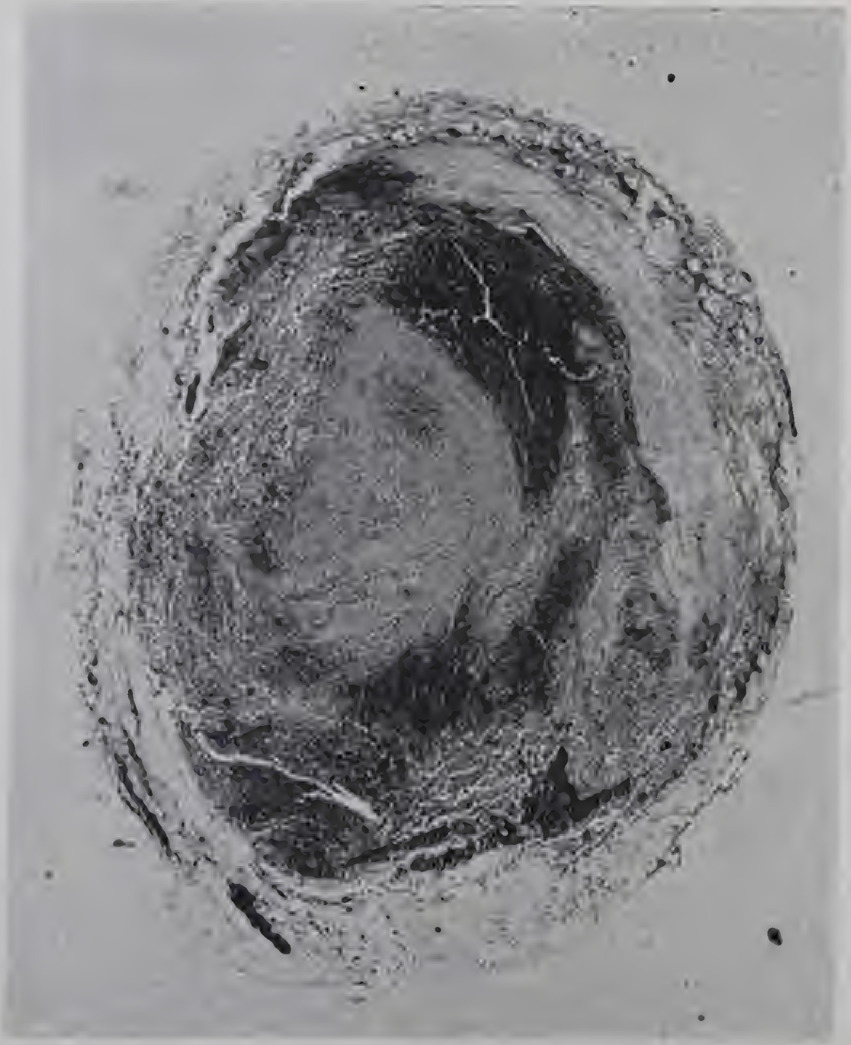

FIG. 2.

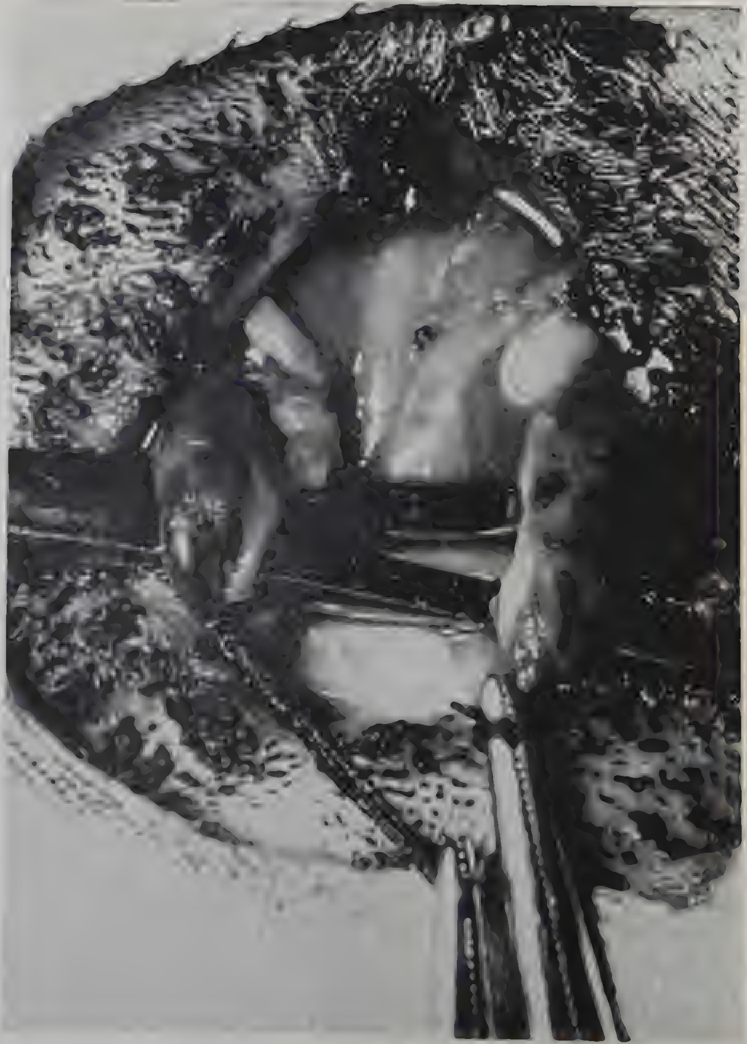

FIG. 1.

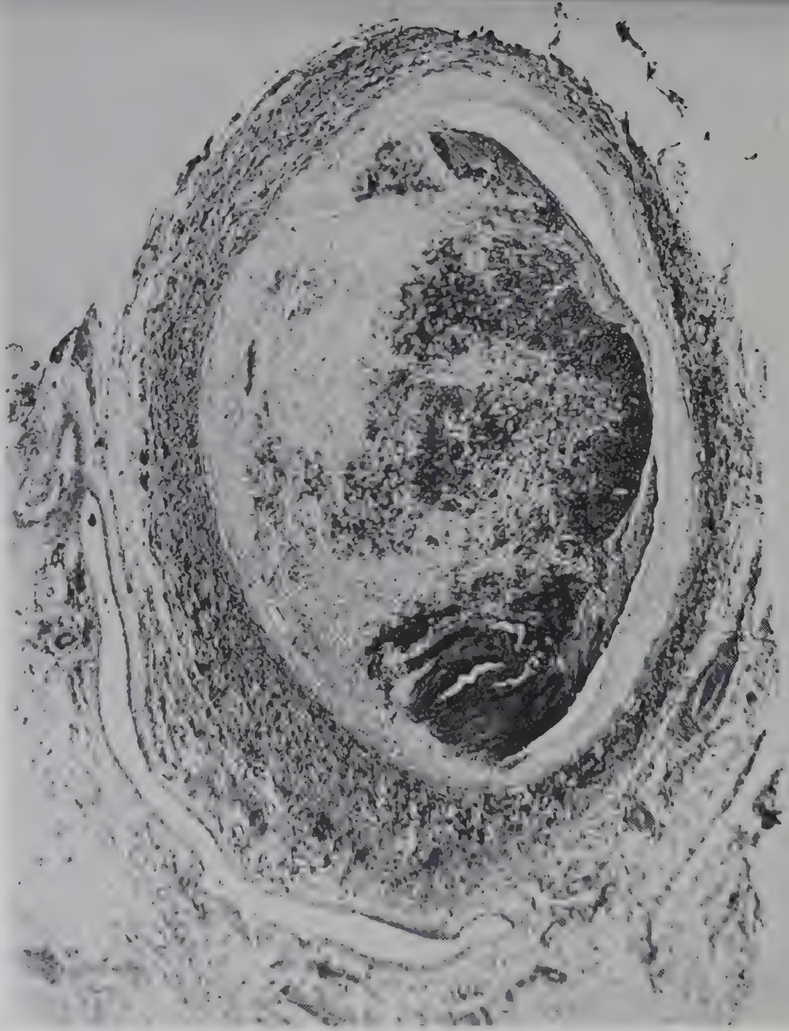

Fig. 4. Microscopic section of Fig. 3 (30 ×) showing organization and recanalization.

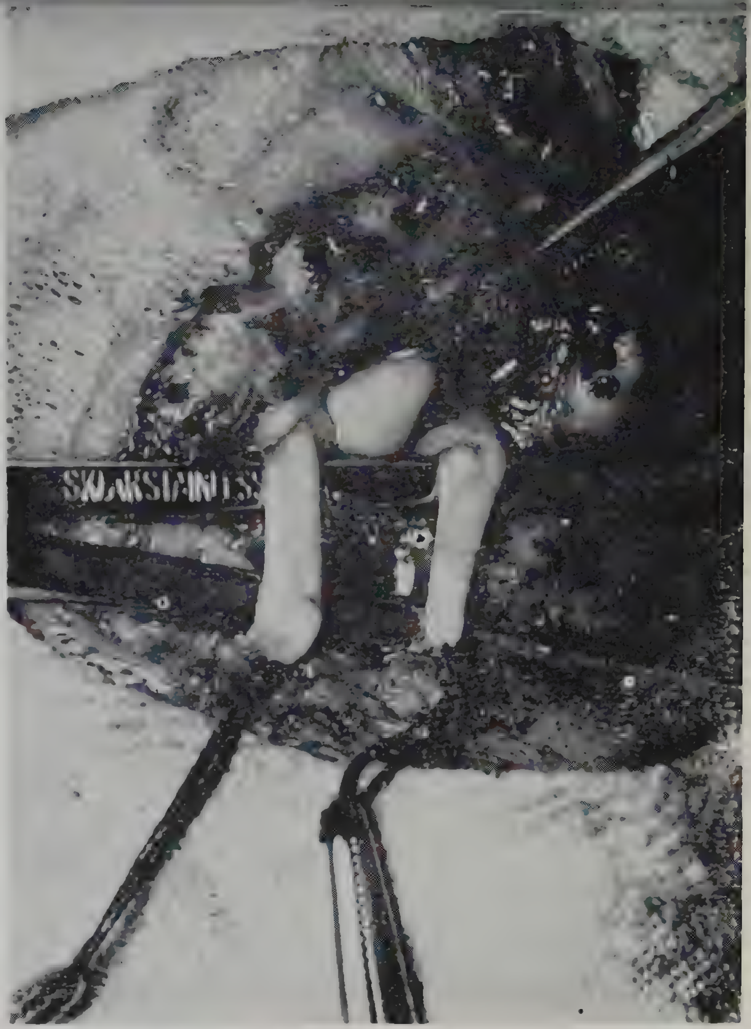

FIG. 3. Jugular veins of rabbit 14 days after ligation and hammering. The veins are thickened and fibrotic.

tion,[†] reveals that they differ in no way from the spontaneous *in vivo* clot seen in aseptic thrombophlebitis. The progressive morphologic changes leading to organization and recanalization are also similar to spontaneous *in vivo* clots (Fig. 1, 2, 3, 4). I the last 140 veins, the procedure has bee successful in every case.

Conclusion. An easily controlled, readil available method of experimental clot pr duction is described. It has been uniforml successful in our hands.

[†] We wish to thank Dr. David M. Grayzel for his interpretation of the microscopic sections.

15677

Viscosity of Normal Human Synovial Fluid.

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(With technical assistance of Audrey F. Grubin.)

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Synovial fluid is a viscous solution containing a longchain mucopolysaccharide (hyaluronic acid), protein and electrolytes.[1] The relative viscosity of such fluid is related to the amount of hyaluronic acid present[2–4] and to the state of polymerization and aggregation of the polysaccharide.[5] When joint fluid is incubated with an hyaluronidase,[6] the viscosity approaches but does not quite reach the viscosity of water.[6] The slight residual increase in viscosity may be due to the proteins present which are unaffected by purified preparations of hyaluronidase. (Isolated pure hyaluronic acid never attains the high viscosity found in native (human or animal) synovial fluids, presumably because the procedures used in isolation disaggregate the polysaccharide as it occurs in the native form.[5] The human knee joint is functionally far different from the 4-legged animal's astragalo-tibial joint from which most specimens of so-called normal joint fluid have been obtained.[3,7] It seemed of interest t determine, if possible, the characteristics o normal human synovial fluid, to compar with pathological fluids which can readily b obtained in large quantity. Reports on th viscosity of normal human synovial flui have yielded wide variations and the technics of viscosimetry are open to some criticism.[8,9] When an apparently normal huma knee joint is tapped at the postmortem table, one can rarely withdraw more than 1 or cc of joint fluid. Thus, 2 to 4 cc of huma knee joint synovial fluid can be obtaine from the 2 knees of an individual, which i less than the capacity of the standard 5 c Ostwald viscosimeter. On casual inspectio it was found that the fluid obtained was ver viscous, and it was probable that a viscosimeter of less than 5 cc capacity would giv an inaccurate reading with such viscous material.

Methods. Viscosities were measured i 5 cc Ostwald viscosimeters in a water bat at temperature of 21° ± 1°C. Total proteins were determined with a gradient tub method.[10] In this laboratory, it has bee ound that the gradient method has a good orrelation with the Howe method in esti- ation of protein content of synovial fluid. has also been shown that the specific gravi- of a 1% solution of purified sodium hy- luronate* is below the scale of the gradient ethod employed and does not materially af- ect specific gravity estimation of synovial uid protein.

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Eight pathological joint fluids of varying iscosity were diluted with 0.9% sodium hloride, the relative viscosity at each dilu- ion was plotted against dilution and curves ere drawn (Fig. 1). It can be seen that he fluids with a very high initial viscosity how an abrupt fall on minimal dilution with

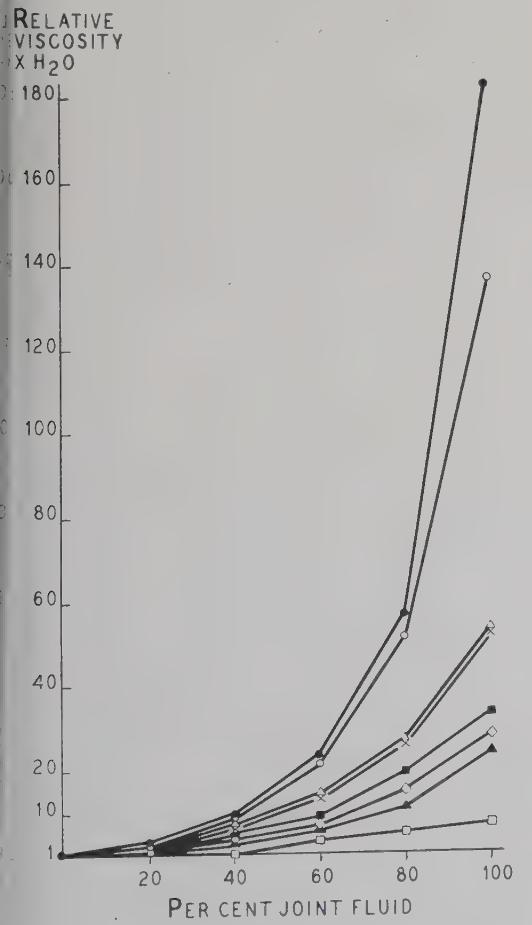

FIG. 1.
Curves of relative viscosity obtained by dilution of 8 pathological joint fluids with 0.9% NaCl.

TABLE I.
The Relative Viscosity and Total Protein Content of Normal Joint Fluids.

Pt.	Diagnosis	Peripheral oedema	Amt removed both knees, cc	Relative viscosity			Total protein
				Dilution, %	Rel. visc. diluted sample	Relative visc. by extrapolation	
C.H.	Carcinoma vocal cords	0	2	40	11.8	>185	—
F.B.	Hypertensive cardiovascular disease	0	2.5	50	36	>185	1.3
R.D.	Lymphosarcoma	0	3	40	8.9	134-184	—
S.McC.	Hypertensive cardiovascular disease, mesenteric thrombosis	0	3	50	88.2	>185	1.5
E.T.	Hypertensive cardiovascular disease	0	3.6	50	16.8	185	2.3
E.C.	Intestinal obstruction	0	3.6	50	12.6	60-134	2.4
H.H.	Carcinoma stomach	0	4	50	12.8	60 134	1.8
F.L.	Coronary occlusion	0	1	20	4.4	>185	—
T.W.	Lung abscess with terminal hemorrhage	0	2	40	10.1	>185	2.0
C.N.	Hypertensive cardiovascular disease	+++	rt. 30	100	—	3.0	1.6
			lt. 20	100	—	7.2	1.7
T.J.	Hypertensive cardiovascular disease	++	rt. 15	100	—	47.0	2.2
			lt. 12	100	—	62.2	2.2
N.S.	Nephrosis	++++	not all removed	50	5.1	7·20	.94

---

* Obtained through the courtesy of Dr. K. Meyer.

TABLE II.
The Relative Viscosity and Total Protein Content of Pathological Joint Fluids.

Pt.	Diagnosis	Knee joint	Amt removed cc	Relative viscosity	Total protein
R.R	Rheumatoid arthritis—old	right	300	175	4.8
E. O'T.	" " —early	"	20	6.8	4.2
L.S.	Torn medial meniscus	left	15	13.8	4.6
H.T.	Rheumatoid arthritis—old	right	60	48.2	5.3
H.F.	" " "	"	40	16.8	4.8
P.R.	" " —early	"	20	5.6	5.9
T.B.	Gonococcal arthritis	"	20	8.4	4.4
A.N.	Arthritis one knee assoc. with lymphogranuloma inguinale	"	75	40.5	4.4
G.N.	Intermittent hydrarthrosis	left	40	11.2	5.9
M.P.	Gonococcal arthritis—old	"	40	8.8	6.0

an elliptical type of curve. This type curve may be superimposed on the dilution curve for pleural fluid reported by Meyer.[4]

It has been possible to approximate the viscosity of normal joint fluid by dilution and extrapolation on these curves This has been done by dilution with 0.9% NaCl and extrapolation on the curves in Fig. 1 (Table I). In normal joints, one factor which is very important is the amount of fluid which can be obtained at tapping. In this small series this seems to be directly related to the amount of peripheral edema present and thus the joint space can be looked upon, as Bauer[11] has felt, as an extracellular space.[†] Where no more than 5 cc was obtained on tapping both knees, the viscosity of the joint fluid was greater than or equal to the viscosity of our most viscous pathological joint fluid. In 2 instances, with rightsided heart failure and marked pretibial edema, but with apparently normal knee joints, a larger amount was withdrawn from the knees (Pt. C.N. and T.J. in Table I), and the viscosity of these was less than that seen in normal fluids. One patient on the ward with generalized anasarca in the nephrotic stage of chronic glomerulonephritis was tapped and 2.5 cc of fluid was removed from his right knee. This was not viscous and on dilution and extrapolation gave a viscosity of 7 to 20 times water. As shown in Table I, in the absence of peripheral edema, the calculated viscosity of normal joint fluid was very great compared to the viscosity of the joint fluid obtained from a random selection of pathological joints shown in Table II. The protein content of fluid from normal joints as measured by a specific gravity method was below 3.0%, while in pathological joints, the protein content of the fluid was above 3.0%. (Table I and Table II).

Discussion. The viscosity of synovial fluid from knee joints of patients without symptoms or signs of joint disease is equal to or in most instances greater than the viscosity of joint fluids obtained from pathological knee joints. Because of the small amount of fluid obtained, the amount of hyaluronic acid present in these normal joint fluids cannot at the present time be determined by chemical means. However, the high viscosity would indicate that these fluids contain either a large amount of hyaluronic acid per unit volume or that the polysaccharide is in a highly polymerized state or in a state of high aggregation. On standing at icebox temperature over a period of weeks in a sterile state, the viscosity of the pathological fluids does not change.[12] Thus, if a specific enzyme is present, it is in an inactive state when removed from the body.

Conclusions. 1. The viscosity of normal human knee joint fluid is greater than that of fluid found in most pathological conditions. 2. The decreased viscosity of joint fluids found in pathological states may be due to one of two factors or to both, (a) dilution with extracellular water, (b) depolymerization or dissociation of a highly polymerized hyaluronic acid by hyaluronidase.

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[†] We would like to visualize this space as the interfibrillar tissue space. In this sense it is extracellular.

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15678

Inactivation of Poliomyelitis Virus in Relation to Gastric and Intestinal Digestion.*

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Whether or to what extent gastric and intestinal digestion inactivates poliomyelitis virus is a problem that has an important bearing on intestinal entry of infection and on the source of virus in the intestinal contents and stools. This problem has not been studied as closely as might be desired in its relation to the conditions of normal digestion, although such studies as are available indicate that the virus displays a considerable degree of resistance to inactivation in the gastrointestinal tract. Thus, Flexner, Clark and Dochez[1] found active virus in the intestine 2 hours after administration by stomach tube; and by the same method of administration Clark, Schindler and Preston;[2] Clark, Roberts and Preston;[3] and Levaditi, Kling and Lepine[4] found active virus in the stools within 48 hours. The effects of H-ion concentration upon virus in the range with which we are here concerned has been studied by Loring and Schwerdt[5] but their experiments were limited to periods of 1-2 hours and were done at room temperature. So far as we know no studies on the inactivating effects of individual digestive enzymes on poliomyelitis virus have been recorded.

In the present experiments the test conditions were intended to approximate those obtaining in the human stomach in respect to temperature, time, pH range, and presence of pepsin. In children over one year of age (the group most susceptible to poliomyelitis), Cutter[6] has shown that the fasting gastric contents 10 minutes after stimulation with histamine display an average acidity of pH 1.5 and occasionally reach pH 1.2. In young adults, the level is pH 1.0-1.2.[7] Hammon and Izumi[8] showed that poliomyelitis virus is not inactivated at levels of pH 4.0 and up. Gastric evacuation begins a few minutes after ingestion.[9] At 4 hours, after ingestion of various types of food, the stomach is nearly empty.[9] On the basis of such data, we have selected for our studies a range of pH 1.0-4.0 and exposure periods of 5 minutes to 4 hours. All observations were made at 37°C, or approximate body temperature.

The Lansing (Armstrong) mouse-adapted strain of poliomyelitis virus was used. Stock 10% and 20% suspensions of infected mouse cord in physiological NaCl solution were clarified at 18,000 r.p.m. for ½ hour, then

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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stored in ampoules on dry ice, and appropriately diluted with saline solution at the time of use. Swiss mice, all, with a few exceptions, of the Webster strain from the Carworth Farms, were employed, using a standard intracerebral inoculum of 0.03 cc. Positive results were determined either by the appearance of paralysis or, in the case of mice found dead without observed symptoms, by histological examination. While differing from the current practice of judging results by the gross fatality rate, our method somewhat increases the accuracy and significance of the results by eliminating deaths from nonpoliomyelitic causes. In the tables, the numerator expresses the number of mice showing poliomyelitis and the denominator, the number of mice tested. pH determinations were made electrometrically, both before and after incubation of each preparation. Mixtures were adjusted with HCl within the range pH 1.0-3.0; for pH 4.0, an acetic acid-NaOH mixture was used. At the end of incubation at 37°C, each preparation was returned to approximate neutrality by the addition of NaOH. In the pepsin experiments, acid solutions of Lilly's pepsin were made up to contain 12% pepsin in the final mixture with virus and adjusted to the desired pH The proteolytic activity of each pepsin preparation was tested on coagulated egg albumin. At the end of each incubation period, enzyme activity was stopped by the addition of NaOH to approximate neutrality. For the controls, pepsin inactivated at 70°C for ½ hour was used in the same amounts as in the tests. (Results shown in Tables I and II).

TABLE I.
Inactivation of Lansing Virus at Varying pH Levels.
1:500 suspensions.

	Incubation time					
pH	5 min	¼ hr	½ hr	1 hr	2 hr	4 hr
1.0	0/7	0/7	0/7	0/7	0/7	0/6
1.5	3/6	0/6	0/6	0/6	0/6	0/6
2.0	6/7	4/7	5/7	4/7	3/6 (0/13)*	0/6
3.0	6/8	6/7	6/8	6/7	5/8 (14/19)*	8/8
4.0	7/8	7/8	4/8	5/8	5/8	6/8

* Another series of tests.

This group of experiments shows complete inactivation of virus in 5 minutes at pH 1.0. At pH 1.5, partial inactivation occurs at 5 minutes, and complete at 15 minutes, while at pH 2.0, partial inactivation begins at about 2 hours and is complete at 4 hours. At pH 3.0 and pH 4.0, no inactivation is found at 4 hours. In other tests not tabulated, inactivation was complete at pH 3.0 in 22 hours, but incomplete at pH 4.0 in the same period.

The data indicate a slightly greater degree of inactivation with pepsin than by acidity alone, but the difference between tests and controls is small. The optimum proteolytic effect of pepsin is stated to occur at pH 2.0-2.8.[10]

Effect of Trypsin. Supplementing the study of gastric digestion on virus, the effect of trypsin was investigated. For technical reasons, mice could not be used for this purpose and the experiments were performed on monkeys with a freshly isolated strain of poliomyelitis virus in its first passage. A suspension made up to contain 10% pancreatin (Parke-Davis) and 10% virus adjusted to pH 7.9 was incubated for 4½ hours at 37°C. The activity of the pancreatin was confirmed by visible digestion of egg albumin and by determination of pH and free acids in casein digest. At the end of incubation pancreatic activity was inhibited by lowering the pH to 4.2 by the addition of HCl. In 6 Rhesus monkeys, 0.5 cc of the virus-pancreatin mixture was instilled daily into each nostril for 5 consecutive days. Three monkeys developed typical paralytic poliomyelitis, and one, nonparalytic poliomyelitis as demonstrated by characteristic lesions in the cord. Two monkeys showed no evidence of infection. Of 6 controls given virus in saline suspension by the same procedure, none developed clinical signs of poliomyelitis and the olfactory bulbs of 2 showed no lesions. It would appear that exposure to trypsin not only fails to destroy the virus but may even increase its infectivity.

Comment. Our experiments indicate tha at high levels of acidity such as prevail in

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[10] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, **2**, 113.

TABLE II.
Inactivating Effect of Pepsin.

	Virus conc.	1:20		1:200		1:500	
pH	Incubation time, hr	Active pepsin	Inactivated pepsin	Active pepsin	Inactivated pepsin	Active pepsin	NaCl-HCl controls*
2.0	½	9/10	10/10	3/10	6/10	9/10	
,,	1	0/10	1/10	0/10	2/10	3/10	
,,	2	0/10	0/10	0/10	0/10	0/10	
3.0	½					9/10	
,,	1					7/10	
,,	2					6/10	9/10

* For other comparative figures with NaCl-HCl-virus mixtures, see Table I.

the normal fasting gastric contents, virus is rapidly and completely inactivated. At somewhat lower levels (pH 2.0-3.0) pepsin has a slight inactivating effect. However, the actual effectiveness of this "barrier" in preventing the passage of virus into the intestine is certainly far from complete since the optimal conditions for inactivation are present in the stomach only at times. The ingestion of milk raises the pH of the contents and maintains it, at least in infants up to 19 months, at 3.2 or more for at least 2 hours.[11] With carbohydrate foods (Ewald test meals) on the other hand pH levels are frequently at the inactivating levels. Thus, Kahn and Stokes[12] found in 5 human subjects aged 4, 5, 6, 9 and 43 years old respectively with gastrostomies that after one hour, the pH was 1.32, 1.56, 1.57, 1.73 and 3.31. At the height of digestion of an ordinary meal containing meat, the acidity of the gastric contents is stated to lie in the range pH 1.3-2.5.[9] Mucus and saliva are said to raise the pH somewhat.[13] The rate of gastric evacuation is another factor of importance. After ingestion of most foods, evacuation begins in 1-7 minutes, and with fluids a considerable amount may leave the stomach almost at once. On the other hand, emptying requires 3 hours or more, being especially rapid with carbohydrates (95% at 3 hours), a little less so with proteins (80-85% at 3 hours) and quite slow with fats (30-50% in 3 hours).[9]

Taking the varying conditions of gastric acidity and motility into consideration it seems clear that when virus is swallowed, some of it is destroyed in the stomach and some enters the intestine intact, but the proportions cannot be well estimated. Conditions of high acidity, such for instance as occur after ingestion of carbohydrates and meats, favor inactivation while highly buffered foods, particularly milk, tend to prevent it. Virus contained in that part of the ingesta which leaves the stomach too soon to be highly acidified is apt to escape inactivation.

Since the pH of the intestine has a range of about 6.0-8.0[14] and since, as we have shown, trypsin has no inactivating effect, any virus reaching the intestine is likely to remain intact, and to have the opportunity of causing infection. Virus can also become concentrated in the large bowel as water is abstracted from the contents and thus become easily demonstrable in the stools. While, as we[15] have mentioned elsewhere, we believe that primary intestinal entry is an exceptional event, we suspect on the basis of the frequency of lesions in the celiac ganglia[15] that secondary intestinal entry following pharyngeal entry is not uncommon.

Summary. At the pH levels prevailing in the stomach when only gastric juice is present, and at the height of digestion of carbohydrate and mixed meals containing meat, poliomyelitis virus is rapidly inactivated. Pepsin contributes slightly to inactivation. Since the pH levels necessary for virus inactivation are present in the stomach only part of the time, and since part of the gastric contents are evacuated before such levels can be attained, a certain proportion of ingested virus has the opportunity to escape intact into the duodenum; here and in the rest of the intestine the pH is too high to inactivate it and trypsin has no inactivating effect. Virus entering the bowel therefore must be presumed to remain active, thus providing conditions suitable for secondary intestinal entry of infection, and also for concentration of virus in the contents of the large intestine.

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